CALCIUM DYNAMICS AND ACTIVE OXYGEN SPECIES IN NEUROTOXICITY

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IN

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BY

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CERTIFICATE

This is to certify that the work presented in this thesis entitled, **"Calcium *Dynamics and Active Oxygen Species in Neurotoxicity*"** has been carried out by **Ms. Sarah Musavi** under my supervision.

She has fulfilled the requirements of the **Aligarh Muslim University** for the degree of **Master of Philosophy** in **Biochemistry.**

The work included in this thesis is original unless stated otherwise and has not been submitted for any other degree.

Phalkar
(Poonam Kakkar)

To

All My Teachers

Who Helped Me Ascend

Each Rung Of the Academic Ladder

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Sarah Musavi

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CONTENTS

PREFACE

The biochemical response of occupational and environmental xenobiotics in different target tissues involves specific molecular loci and mechanisms in toxic tissue injury. However, two general events, namely, oxidative stress caused by free radicals and destabilisation of calcium homeostasis are observed in most cases. The relation between these two mechanisms has been the subject of research in recent years Earlier work done in this laboratory indicated the following central unspecific pathway in toxicity ie., xenobiotics---------> free radicals -------> membrane damage ---------> altered calcium functions -------> diversity of toxic effects (Kakkar *et al.* 1992a, 1993, 1995). Conditions of neurotoxicity offer ideal systems to test this aspect since many neurotoxicants cause oxidative damage to membranes and derangement of calcium functions. Brain is especially vulnerable to oxidative damage due to its high content of membranous structures, rich polyunsaturated fatty acid availability, oxgyen availability and atypical antioxidant defenses. Role of oxidative stress has been studied in a number of neurodegenerative disorders, such as Parkinson's and Alzheimer's disease, ischemia/reperfusion, stroke, neuronal damage and neurogenesis etc. Involvement of free radical related processes and the modulation of antioxidant defenses during mild anaesthesia by ether and phenobarbitone has been reported

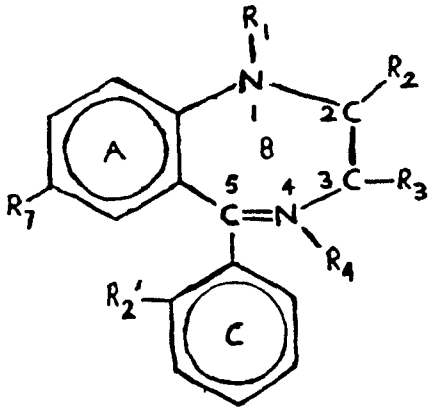
earlier (Awasthi *et al.*, 1989; Viswanathan, 1995, 1996). In view of this response of other neuroactive substances to oxidative phenomenon, assumes significance. Therefore, Benzodiazepines, especially diazepam which have sedative - hypnotic properties but have low potency for fatal CNS depression was chosen to study its effect on prooxidant/antioxidant system alongwith its interelation to altered calcium functions of different regions of rat brain. In the first phase the involvement of free radical processes in the neurotoxicity of diazepam has been investigated as the objective of this study, and the results therefore constitute this dissertation.

BACKGROUND LITERATURE

Diazepam

Diazepam(DZ) belongs to the group of 3000 and odd benzodiazepines (BZDs) out of which 120 have been tested for biological activity and 35 are in clinical use in various parts of the world. In 1976, the most popular drug prescribed in the U.S.A was diazepam (Valium).

Benzodiazepines (Fig. 1) are not a general neuronal depressant as are the barbiturates and it is thought that the action of BZDs are a result of potentiation, of the neuronal inhibition that is mediated by GABA (Gilman *et al.*, 1991). These agents are widely prescribed in the treatment of anxiety and nervousness. Most of the BZDs used clinically have high anxiolytic potential and low potency as general depressants of CNS function. They have the general ability to relieve symptoms of anxiety with minimum interference with cognitive function or wakefulness. Nevertheless, the BZDs all possess sedative- hypnotic properties to varying degrees and are preferentially used over barbiturates mainly because of their low potency to produce fatal CNS depression.



BZDs	R_1	R_2	R_3	R7	R_2'
Diazepam	$-CH_3$	=O	-H	-Cl	-H
Clonazepam	-H	=O	-H	$-NO_2$	-Cl
Flurazepam	$-CH_2CH_2N(C_2H_5)_2$	=O	-H	-Cl	-F
Nitrazepam	-H	=O	-OH	-Cl	-H

Fig. I: The general structure of benzodiazepine (Gilman *et al.*, 1991).

The term BZD refers to the portion of the structure composed of a benzene ring (A) fused to a 7.membered diazepine ring (B). However, since all the important BZDs contain a 5-aryl substituent ring (C) and a 1,4-diazepine ring, the term has come to mean the 5,aryl-1,4-Benzodiazepenes. Various modifications in the structure of the ring systems have yielded compounds with similar activities.

A large number of congeners with very little variations have come in the market since the introduction of diazepam. Although, their pharmacologic effects do not differ greatly in terms of their clinical application, there are some differences in their major routes of biotransformation, volumes of distribution and half lives with respect to their pharmacokinetics in overdose eg., chlordiazepoxide and oxazepam have shorter half lives than diazepam and flurazepam. Therapeutic levels of diazepam or chlo-rdiazepoxide are reported to be about 0.5mg/100ml. Diazepam and most of the BZDs do not cause muscle relaxation in man, in nonsedative doses. Diazepam is a GABA enhancing drug which can act as a neuroprotective agent following ischemic insults (Schwartz *et al.*, 1994)

The depressant effects of DZ on chemoreceptor pressure reflex and depressor reflex have been suggested to be mediated by activation of the GABA receptors in the rostral ventrolateral medulla (Gu *et al.*, 1993). Benzodiazepine agents are widely prescribed in the treatment of anxiety and nervousness. At concentrations of about 1 um, BZD can inhibit calcium currents and calcium dependent release of neurotransmitters. The active metabolites of DZ bind to plasma proteins depending upon its lipid solubility and are rapidly absorbed and delivered to highly perfused tissues including the brain. Sedative or hypnotic drugs when used in high doses, can induce general anaesthesia except BZD.

While the BZDs in clinical use exert qualitatively similar effects, yet there are varying patterns of their therapeutic application depending on their pharmacodynamic spectra and pharmacokinetic properties. A number of distinct mechanisms of action contribute in varying degrees to sedative hypnotic, muscle relaxant, anxiolytic and anticonvulsant effects of BZD. Patients ingesting BZDs on a chronic basis (40-80 mg or more/day for 1-2 months or more) may exhibit mild to moderate symptoms of withdrawal, with severe withdrawal symptoms in those patients taking the drug for many months to year. In most cases ingestion of BZD agents of up to 1.5 g results in only minor toxicity ie. CNS depression. Fig II shows the various reported effects of BZD on CNS

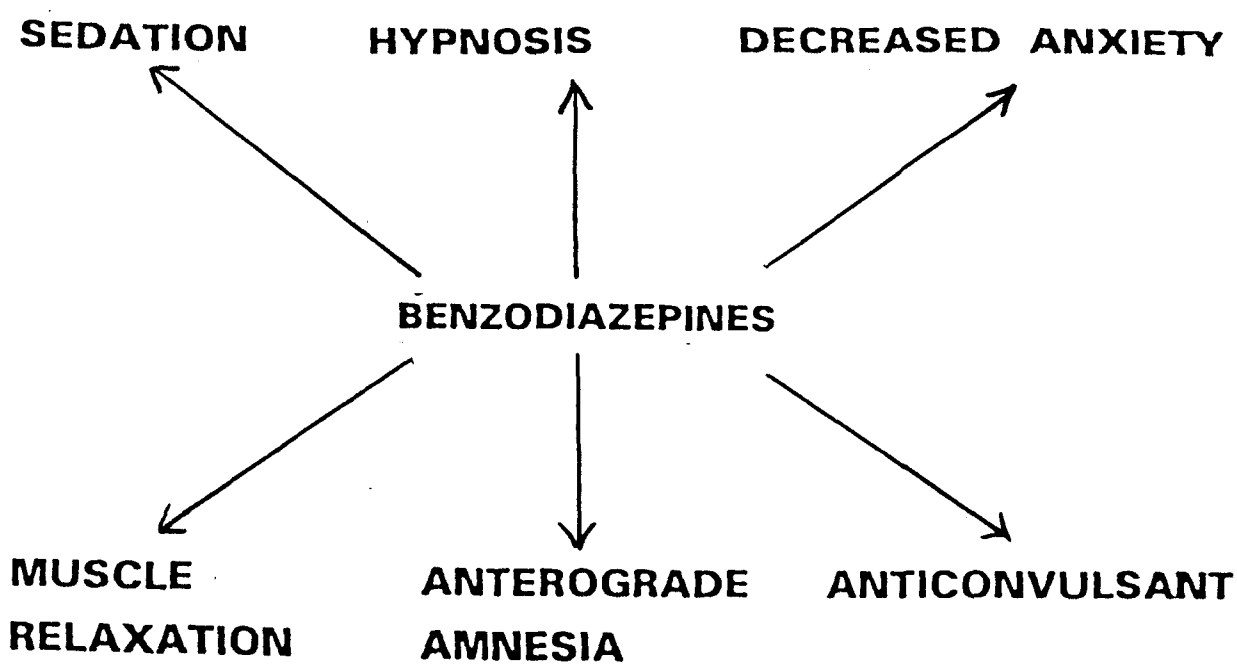


Fig. II: Various reported effects of BZDs on CNS.

Studies on metabolism of diazepam and related BZDs by human liver microsomes *in vitro* showed that the use of organic solvents or small quantities of HCl, inspite of enhancing the solubility of DZ produced a substantial inhibition of its metabolism by both major pathways (N-demethylation and C3- hydroxylation). In the absence of any of these solvents, formation of N-demethyldiazepam increased approximately linearly with diazepam concentration to 200um, and did not show saturation (Hooper *et al.*, 1992), thus highlighting some methodological problems inherent in *in vitro* metabolism studies with BZD and throwing light on the metabolism of DZP *in vitro* by human liver. Diazepam induced anterograde amnesia for inhibitory avoidance training is mediated through influences involving the basolateral amygdala nucleus (Tomaz *et al.*,1992). Contingent tolerance developed in rats which received intermittent BZD treatment before, but not after amygdala stimulation (Tietz, 1992) Administration of diazepam to male or female mice at estrus or diestrus showed that concentrations of diazepam in the brain after i.p. injections were not influenced by stage of the estrus cycle (Carey *et al.*, 1992).

Benzodiazepine Receptors

Drug receptors are special proteins in that they incorporate the dual properties of recognition and transduction (Kenakin *et al.*, 1992). Thus, they exist to recognize endogenous agonists and transmit the messages they contain to their cellular hosts (Kenakin *et al.*, 1995). In general, biologically active ligands have both the ability to bind to receptors and the potential ability to modify receptors for subsequent interaction with membrane proteins (Colquhoun, 1987).

The psychoactive drugs, benzodiazepines bind to specific receptors on the membranes of rat brain cells, suggesting that there may be an unknown endogenous neurotransmitter which is the natural ligand for the BZD receptor. The binding sites are distributed unevenly through the brain (Squires and Braestrup, 1977). The diazepam receptor described by Squires and Braestrup seems to be physiologically significant since it can be correlated to pharmacological activities.

The principal site of action of BZDs-one that makes this class of drugs the leading anxiolytic compounds - is a domain that allosterically regulates chloride channel gating by GABA on GABAa receptors (Pritchett *et al.*, 1989). Because of the initial identification of BZD receptors outside the CNS, this class of recognition sites became commonly known as **`peripheral type benzodiazepine recognition sites'** (Braestrup

and Squires, 1977). These BZD binding sites exist in nearly all mammalian tissues, including the CNS, where their density is either nearly comparable to or greater than that of the BZD recognition sites of $GABA_A$ receptors (Krueger and Papadopoulos, 1992). Peripheral type BZD recognition sites **(PBRs)** are found predominantly on mitochondria. **Mitochondrial BZD receptors** (MBRs) were first discovered because they bind the BZD, diazepam with nanomolar affinity (Braestrup and Squires, 1977). Studies have revealed that the two classes of BZD recognition sites display distinct structural specificities (Wang *et al.*, 1984). In rodents, diazepam is rather non selective, binding with nearly equal affinity to both classes of sites i.e., MBRs and $GABA_A$ receptors.

The fact that rodent species exhibit the highest affinities for BZDs permitted the initial discovery of MBRs, hence their classification as a BZD recognition site (Anholt *et al.*, 1985). Now it is clear that the BZD binding property of MBRs is at best weakly conserved across species. MBRs are found abundantly in tissues involved with electrolyte transport, (Benavides *et al.*, 1983) but are expressed at relatively low levels in much of the brain, as also in skeletal muscle and gastrointestinal tract, but it is emphasised that although these tissues have low levels of MBRs, the densities of receptors that can be found are still quite high in comparison with other receptors (>500 fmol/mg of protein) (Papadopoulos *et al.*, 1991). MBRs are localized differently in different tissues, or they may be preferentially localized at contact sites of outer and inner mitochondrial membranes (Hirsch *et al.*, 1989a; Mukherjee and Das, 1989).

Ligands binding to MBRs have been found to alter mitochondrial respiratory control (Hirsch *et al.*, 1989b). Barbiturates and BZDs enhance GABA receptor currents (Macdonald and Barker, 1979) but through different allosteric regulatory sites on the GABA receptor. Each drug interacts with a different site on the GABA receptor (Macdonald and Olsen, 1994) (Fig. III).

PBRs are distinct from the central $GABA_A$/BZD receptors in their drug specificity, anatomical distribution, subcellular localization and function (Gavish *et al.*, 1992). PBRs in mammalian brain are predominantly localized in astroglial cells (Sher and Machen, 1984).

Any structural disturbance to membrane components (protein or lipid) may have important consequences on receptor functioning. When ROS occur at exaggerated levels during pathophysiological processes, structural as well as functional disturbances consecutive to oxidative damage are produced (Courtiere *et al.*, 1991). Reduced density

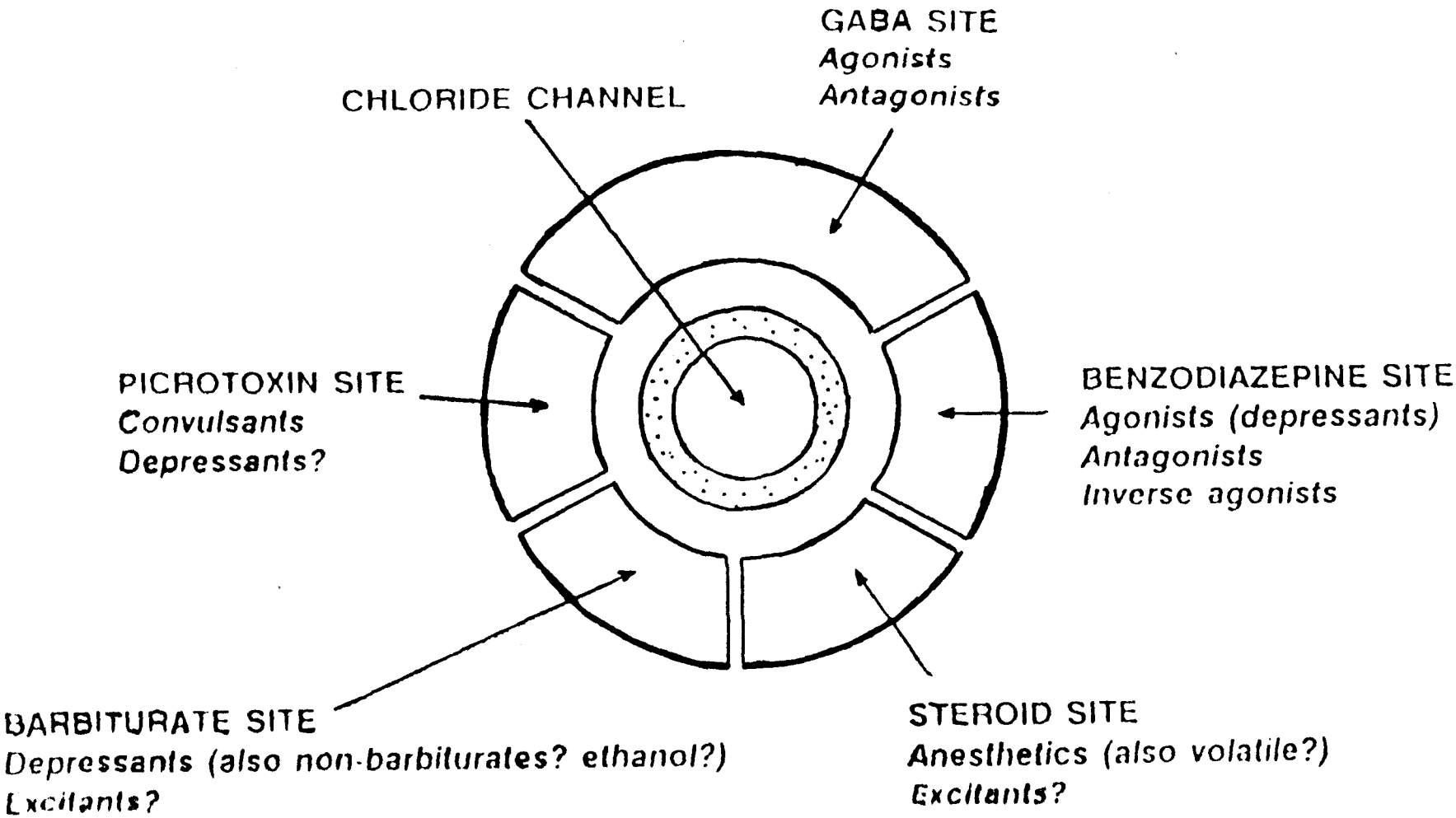


Fig. III: Schematic "donut" model of the GABA receptor-chloride ion channel complex. Each of the sections represents a distinct functional binding domain for the drug class indicated, plus the chloride channel in the middle (Olsen *et al.*, 1991).

of receptors might be a consequence of "down regulative" events, but may also be due to direct degradative processes on receptors themselves or to peroxidation of their lipid environment. The BZD receptor, which is linked to GABA receptor is affected by oxygen radicals (Vliet and Bast, 1992). Considering the consequences of drug-binding, PBRs of liver were found to be more affected by peroxidative events than CBRs of the cerebral cortex (Courtiere *et al.*, 1995).

Free Radical Processes and oxidative stress

A free radical is any species capable of independent existence (for however brief a period) that contains one or more unpaired electrons (Halliwell and Gutteridge, 1989). Examples are superoxide ($O_2^{\cdot-}$, an oxygen centred radical), singlet oxygen (O_2^1), OH (hydroxyl radical), OOR (peroxyl radical), ROH alkoxyl radical thiol (alkoxyl radical) (RS , a sulphur-centered radical) and nitric oxide (NO) etc.

The term reactive oxygen species (ROS) is a collective one that includes not only oxygen centred radicals such as superoxide and hydroxyl (OH) but also some non-radical derivatives of oxygen, such as hydrogen peroxide (H_2O_2), singlet oxygen etc. OH radical is a highly reactive species that can attack all biological molecules, usually setting off free radical chain reactions (Halliwell and Gutteridge, 1989, 1995). If a reaction is thermodynamically feasible, its reaction rate depends primarily on the concentrations of the reacting partners. Thus,to evaluate effects of ROS on biomolecules, their concentrations and sites of production have to be considered, (Gotz *et al*, 1994). Since the major oxidative processes of the cell take place in the mitochondria, the presence of several electron carriers and PUFA rich membrane make this organelle highly susceptible to the attack of active oxygen species (Mehrotra *et al.*, 1991; Kakkar *et al.*, 1992a). Dearrangement of intracellular ion concentration occurs as an early response to injurious stimuli in many cells and tissues (Trump and Berezesky, 1989; Nicotera *et al.*, 1992; Kakkar *et al.*, 1993, 1995) and such activated oxygen species are increasingly recognised to be the mediators of cell injury in many human diseases.

Prolonged oxidative stress incidences associated with depletion of the energy reserve will compromise clearance of excitotoxic amino acids in nervous tissues and may thus be considered a common cause of neurotoxicity under a wide variety of

pathological conditions (Mc Donald and Johnston,1990). Nerve (and muscle) cells are particularly sensitive to disturbance of mitochondrial functions (DiMauro and Devivo,1989). An established toxin - sensitive portion of the energy- generating intracellular catalytic system is the coupling of NADH oxidation and AMP/ADP phosphorylation.

Several different structures that represent interfaces between brain tissue and blood are able to produce superoxide anions during a NADPH-dependent redox cycling of various blood- borne molecules. As these free radicals can alter the functional properties of cell membranes, superoxide formation may promote damage to choroid plexus and cerebral microvessels cells, resulting in the disruption of some properties of the blood-brain and blood cerebro-spinal fluid barriers, possibly resulting in cerebral dysfunctions (Lagrange *et al.*, 1994).

Superoxide radical ($O_2^{\cdot-}$) is much less reactive than $OH^{\cdot}$ (Panasenko *et al.*, 1995). but a number of biological targets can be attacked by it. Though, because of charge restriction, it cannot cross the membranes easily, except RBC membranes but in the presence of transition metal ions, it can give rise to highly reactive OH radical due to Fenton type or Haber-Weiss reaction (Halliwell, 1992).

Fenton Reaction:

$M^{n+} + H_2O_2$ -------> $M^{(n+1)+} + OH^{\cdot} + OH^-$ (M is the transition metal)

Haber-Weiss Reaction:

$Fe^{3+} + O_2^{\cdot-}$ ----------> $Fe^{2+} + O_2$

$Fe^{2+} + H_2O_2$ ----------> $Fe^{3+} + OH + OH^-$

Net $O_2^{\cdot-} + H_2O_2$ ----------> $O_2 + 6OH^{\cdot} + OH^-$

Its interaction with nitric oxide ($NO^{\cdot}$), a free radical produced by several cell types, especially phagocytes and vascular endothelial cells, to give peroxynitrite has attracted much attention in recent years (Saran *et al*, 1990; Huie and Padmaja, 1993). It is now accepted that free radicals, especially active O_2 centred radicals: $OH^{\cdot}$, alkoxyl and peroxyl radicals, attack lipids, carbohydrates, proteins and DNA to induce membrane damage, protein modification (Stadtman and Oliver, 1991; Shacter *et al.*, 1994), enzyme inactivation (Oliver *et al.*, 1990) and strand break (Halliwell and Aruoma, 1993) etc.

which eventually cause a variety of pathological events, cancer and aging (Sies, 1991; Sohal and Dubey, 1994). Fig. IV shows formation of active oxygen species and antioxidant defenses against them.

Superoxide may also attack enzymes containing an iron- sulphur cluster, such as the bacterial enzymes, dihydroxyacid dehydratase, aconitase and 6- phosphogluconate dehydratase (Imlay and Fridovich,1991). Methionyl residues in proteins are quite often the target of free radical attack (Vogt. 1995). It may also inactivate the NADH dehydrogenase complex of the mitochondrial electron transport chain (Zhang *et al*, 1990). On the whole, however, $O_2^{\cdot}$ and H_2O_2 have limited chemical reactivity. Interest has, therefore, focused on their ability to generate more reactive species, such as $OH^{\cdot}$ *in vivo* (Halliwell and Gutteridge, 1990; Minotti, 1993).

Oxidative stress occurs when the production of free radicals overwhelms the body's natural antioxidant defense systems, resulting in oxidative damage of the cells (Sies, 1985), thereby harming that particular cell or tissue (Ryan and Aust, 1992) (Fig. V). ROS-mediated damage leads to pro-oxidant conditions. Such pro-oxidants may then contribute to the development of a wide range of disorders of the nervous system, including Alzheimer's (Smith *et al.*, 1995) and Parkinson's diseases (Duthie *et al.*, 1994). Oxidative cell injury is usually accompanied by perturbations of thiol homeostasis i.e., depletion of soluble thiols, mainly glutathione (GSH),and protein-bound thiols (Reed, 1990). Another intriguing aspect of reactive oxygen toxicity is the ability of superoxide anion as well as redoxcycling xenobiotics to release iron from ferritin, thereby promoting the generation of $OH^{\cdot}$ radicals and iron- oxygen complexes (Reif,1992).

Imposition of oxidative stress upon mammalian cells leads to several metabolic dysfunctions, including DNA damage, depletion of ATP, GSH Oxidation, rises in intracellular free Ca^{2+} (Choi, 1995), enzyme inactivation and lipid peroxidation (Orrenius *et al.*, 1989). All inflammatory diseases impose oxidant stress on affected tissues via the superoxide- producing NADPH oxidase of phagocytes (Kakkar and Viswanathan, 1992). In fact, the spectrum of pathological conditions in which the involvement of active oxygen species has been indicated grows broader and broader (Rodenas *et al.*, 1995). ESR studies have demonstrated the formation of oxygen derived free radicals in the brain (Zini *et al*, 1992) and there is evidence of free radical induced LPO following cerebral ischemia-reperfusion (Floyd,1990) There is also evidence to indicate that free

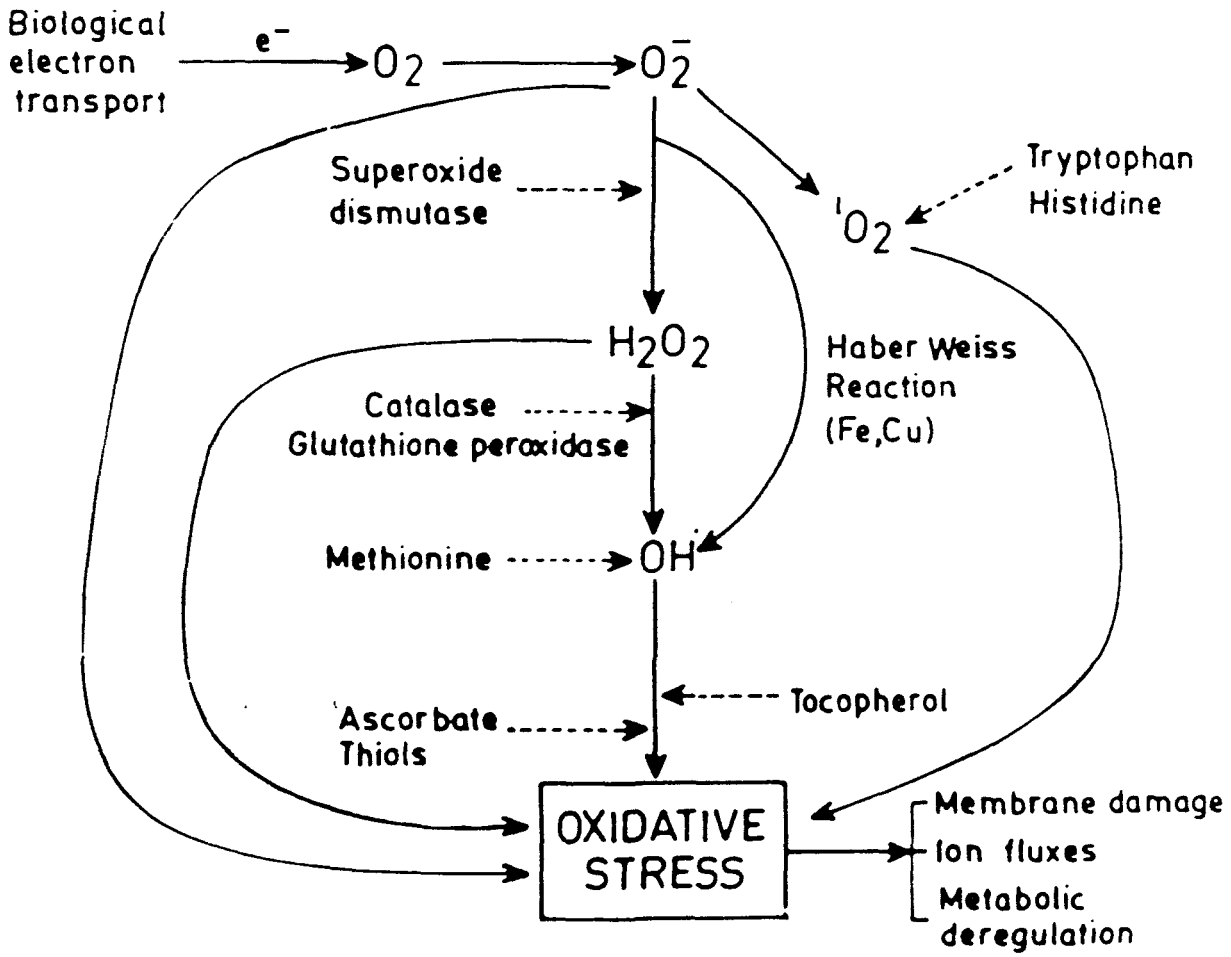
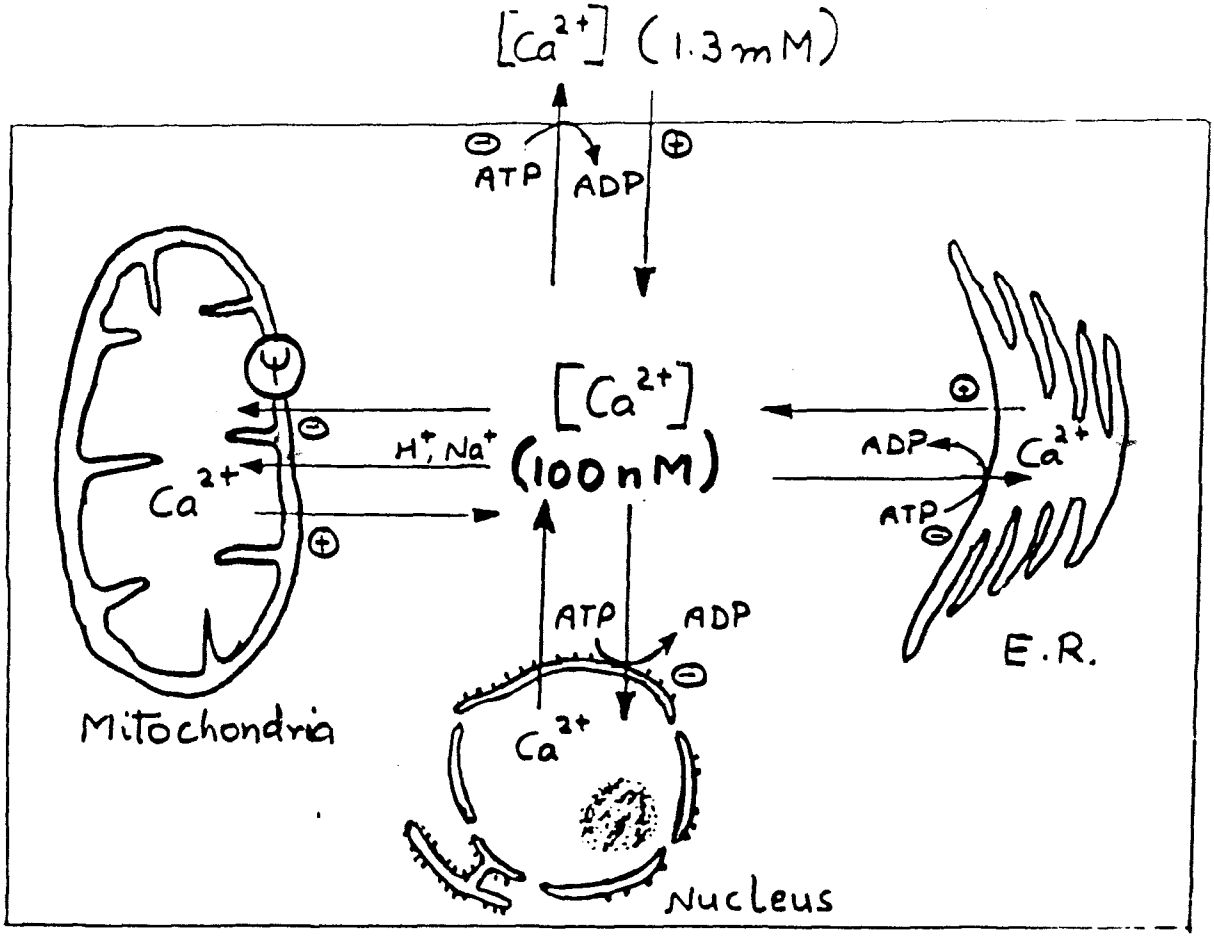


Fig. IV: Formation and scavenging of active oxygen species (Kakkar *et al.*, 1995).


[Ca2+] (1.3mM)
ATP
ADP
[Ca2+]
(100nM)
H+, Na+
Ca2+
ADP
Ca2+
ATP
ATP
ADP
Ca2+
Mitochondria
E.R.
Nucleus

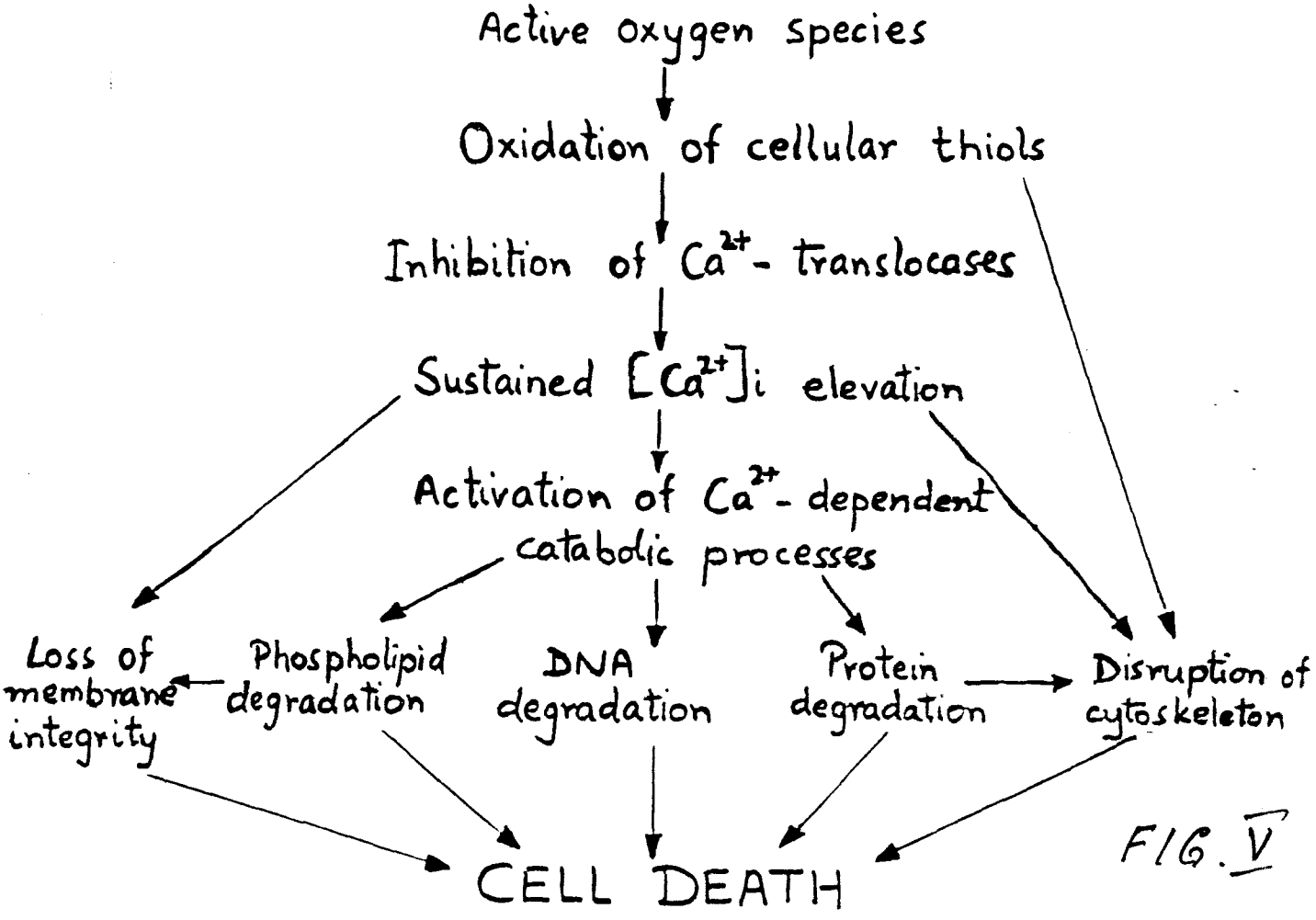

Active oxygen species
Oxidation of cellular thiols
Inhibition of Ca2+ - translocases
Sustained [Ca2+]i elevation
Activation of Ca2+ - dependent catabolic processes
Loss of membrane integrity
Phospholipid degradation
DNA degradation
Protein degradation
Disruption of cytoskeleton
CELL DEATH

FIG. V

radical scavengers and antioxidants can protect against ischemia- reperfusion damage (Halliwell,1992).

Mitochondria as a source of free radical generation

Free radicals are formed during the mitochondrial electron transport (Paraidathathu *et al*,1992) and the rate of ($O_2^{\cdot-}$) formation is proportional to mitochondrial oxygen utilization. Considerable amounts of $O_2^{\cdot-}$ are produced when the electron flow is inhibited (antimycin or rotenone). Whether $O_2^{\cdot-}$ formation is coupled with auto-oxidation of ubisemiquinone or with auto oxidation of Cyt b566 is still unclear,but the latter hypothesis is favoured (Nohl and Stolze, 1992; Gotz *et al.*, 1994). In contrast, ubiquinone has been shown to act as a potent protectant against free radical damage to subcellular membranes *in vitro* (Ernster *et al* 1992). It is assumed that under `normal' conditions little of the $O_2^{\cdot-}$ formed escapes the mitochondria due to the high levels of MnSOD within the matrix. However, during aging, decreased levels of GSH and cytochrome aa3 were measured in brains from old rats (Benzi *et al*, 1992), supporting the theory of increased oxidative stress due to $O_2^{\cdot-}$ production of the respiratory chain as one of the several causes of cell aging (Sohal and Sohal, 1991). Endogenous, or exogenous inhibitors of the mitochondrial electron transfer chain could cause a continuous chronic oxidative stress to mitochondria, finally leading to cell death. Thus, it seems reasonable to assume that a decrease in enzymic activity in the electron transfer chain, due to a decreased formation of enzymes or due to inhibitors, probably results in a chronic decrease in ATP levels and an increase in $O_2^{\cdot-}$ formation. (Gotz *et al*,1994)

Since mitochondria are known to be important cellular sites of both ROS production and oxidative damage by these species (Vercesi and Hoffman, 1993) studies indicate that the mitochondrial inner membrane becomes very sensitive to the deleterious effects of Ca^{2+} when the steady-state levels of the mitochondrial reducing power, NAD(P)H and GSH are decreased by oxidants such as oxaloacetic acid, acetoacetate,t-BHP or diamide (Gunter and Pfeifer, 1990) and the formation of a proteinaceous pore, mediated by a mechanism involving Ca^{2+} and ROS occurs (Szabo and Zoratti, 1992) which is thought to be controlled by redox changes of thiol groups and protein polymerization (Valle *et al*, 1993)

Mammalian mitochondria are highly sensitive targets of the cytotoxic effects of superoxide ($O_2^{\cdot-}$) and nitric oxide (NO). In turn, when $O_2^{\cdot-}$ and NO are simultaneously

produced, they rapidly react with each other to yield the highly oxidizing peroxynitrite anion ($ONOO^-$) which may also be toxic to mammalian mitochondria (Radi *et al*, 1994). While mitochondria are an important cellular source of oxygen radicals (Radi *et al*, 1993), they also represent a preferred intracellular target of free radical attack. Increased mitochondrial free radical production is observed under diverse pathological conditions and is associated with impairment of mitochondrial structure and functions. O_2^- radical attack of mitochondria results in mitochondrial swelling, inhibition of respiration, lipid peroxidation, depletion of mitochondrial antioxidants and dissipation of Ca^{2+} gradients (Zhang *et al*, 1990; Mehrotra *et al.*, 1991, 1993; Kakkar *et al.*, 1996).

The production of partially reduced oxygen species by the mitochondrion has been known for nearly two decades (Boveris and Chance 1973; Turrens and Boveris 1980), whereas the understanding of precisely how and where this free radical production occurs is more recent.The rate of superoxide production by the mitochondria increases when the concentration of oxygen is increased or the respiratory chain becomes largely reduced (Turrens *et al*, 1982). Mitochondria produce superoxide anions at two sites in the electron transport chain. The first site is the ubiquinone to cytochochrome C1 step, which pass through the intermediate ubisemiquinone (Turrens *et al*, 1985). Ubisemiquinone is capable of reducing oxygen to superoxide.The second site of superoxide anion formation is the NADH dehydrogenase (Turrens and Boveris, 1980).

Several reports indicate that the inhibition of the mitochondrial respiratory chain may be caused by the uptake of calcium (Vlessis and Mela Riker, 1989). Mitochondria have been shown to accumulate calcium electrogenically, using the H^+ gradient for this purpose instead of producing ATP, even in the presence of ADP. Calcium overload of mitochondria may activate phospholipases or proteases, either of which may cause uncoupling and inhibition of respiration.

Nitric Oxide in Brain and Oxidative Stress

The free radical gas nitric oxide (NO) is a recently identified neuronal messenger that carries out diverse signalling tasks in both the central and peripheral nervous systems (Kolios *et al.*, 1995). Whereas most neurotransmitters are packaged in synaptic vesicles and secreted in a Ca^{2+}-dependent manner from specialized nerve endings, NO is not , but rather diffuses from its site of production in the absence of any specialized release machinery. NO, being a gas and extremely membrane permeant, can bypass normal

signal transduction routes involving interactions with synaptic membrane receptors (Schuman and Madison, 1994).

$NO^{\cdot}$ is known to be produced *in vivo* by vascular endothelial cells, by phagocytes, and by some cells in the brain (Halliwell and Cross, 1994). One role of NO in the brain may be as a neuromodulatory substance, analogous to some neurotransmitters e.g. NO is reported to influence ion currents through the N- methyl-D-aspartate (NMDA) receptor channels that usually require depolarization (Mayer and Westbrook, 1987) to flux Ca^{2+} (Ascher and Nowak, 1988). Thus, by modulating current flow through this particular channel, NO could potentially influence many Ca^{2+}- regulated neuronal processes that utilize this receptor such as synaptic transmission, plasticity, neurotoxicity, and some aspects of development (Lei *et al.*, 1992).

$O_2^{\cdot-}$ reacts fast with NO in a free radical addition reaction (Saran *et al.*, 1990).

$$O_2^{\cdot-} + NO^{\cdot} \text{ ----------> } ONOO^{-}$$

Peroxynitrite

$ONOO^{-}$ might be directly toxic to cells (Radi *et al.*, 1990). It might also decompose to form a range of toxic products, including some $OH^{\cdot}$ (Hogg *et al.*, 1992).

$ONOO^{-} + H^{+}$ ------> $OH^{\cdot}$, $NO_2^{\cdot}$, NO_2^{+} (Gerschman *et al.*, 1954).

Yet some experiments suggest that $NO^{\cdot}$ may protect against oxidative damage even when $O_2^{\cdot-}$ is being generated (Jessup *et al.*, 1992). Cooperation between nitric oxide and $O_2^{\cdot-}$ has been shown during hypoxia/reoxygeneration-induced neuron injury (Cazevieille *et al.*, 1993).

Considerable controversy surrounds the role of NO in various forms of neurotoxicity that possess different etiologies. One possible consequence of the NO-mediated reduction, in NMDA currents could be attenuation of NMDA mediated neurotoxicity (Hyman *et al.*, 1992).

Dawson *et al.* (1991) have shown that inhibition of NO production can have profound effects in preventing glutamate and NMDA-mediated death of cultured cortical neurons. This and several other studies suggest that NO promotes and is necessary for glutamate-mediated cell death. Contrary to this, other data show that NO can prevent NMDA-mediated cell-death (Lei *et al.*, 1992). In rats, chronically treated with nitric oxide synthase (NOS) inhibitors, no decrease in NMDA toxicity could be detected (Lerner-Natoli *et al.*, 1992).

Thus, considerable amount of data support a role for NO in neurotoxicity ranging from protective to no role to toxic. Different cell populations may be differentially sensitive to NO. Secondly, there may be multiple pathways that mediate glutamate-induced toxicity. NO may participate in neurotoxicity, but neurodegeneration could still proceed in the absence of NO via a parallel redundant mechanism. Third, NO may itself exert multiple, perhaps opposing actions, depending on the timing or concentration of its application.

Thus, NO is an important signalling molecule in a variety of physiological and pathophysiological processes. The observation that neuronal NOS requires both Ca^{2+} and calmodulin for its activity raises the possibility that NO may function in many other systems where rises in intracellular Ca^{2+}, particularly those contributed by NMDA receptors, are known to act as a triggering step.

Thiol Homeostasis

Another important aspect of oxidant mediated cell injury is the disturbance in thiol homeostasis thereby leading to oxidative stress. By one definition, a shift in GSH/GSSG ratio in favour of latter can lead to reductive stress. Oxidised glutathione (GSSG) is formed largely through the reduction of hydrogen peroxide or other hydroperoxides by glutathione peroxidase (Flohe, 1989) (Fig. VI).

GSSG is formed intracellularly through reduction of H_2O_2 or lipid hydroperoxides (LOOH), produced by eicosanoid or lipoxygenase enzyme activity or through nonenzymatic lipid peroxidation. The GSSG thus formed is reduced back to GSH (glutathione) by glutathione reductase,undergoes thiol/disulfide exchange with protein thiols (PSH), or is exported from the cell to plasma, lymph, or in the case of liver, also to bile. The intra-and extracellular fluid concentrations of GSH and GSSG thus reflect the dynamics of these several processes as well as synthesis, hydrolysis and other means of clearance.

Although a majority of the GSSG formed intracellularly is reduced back to GSH by glutathione reductase, most cells export a fraction of the GSSG. (Adams *et al*, 1983). Nevertheless , increased concentrations of GSSG in plasma, lymph and bile have been demonstrated in response to a number of acute oxidants *in vivo.* (Smith, 1991)

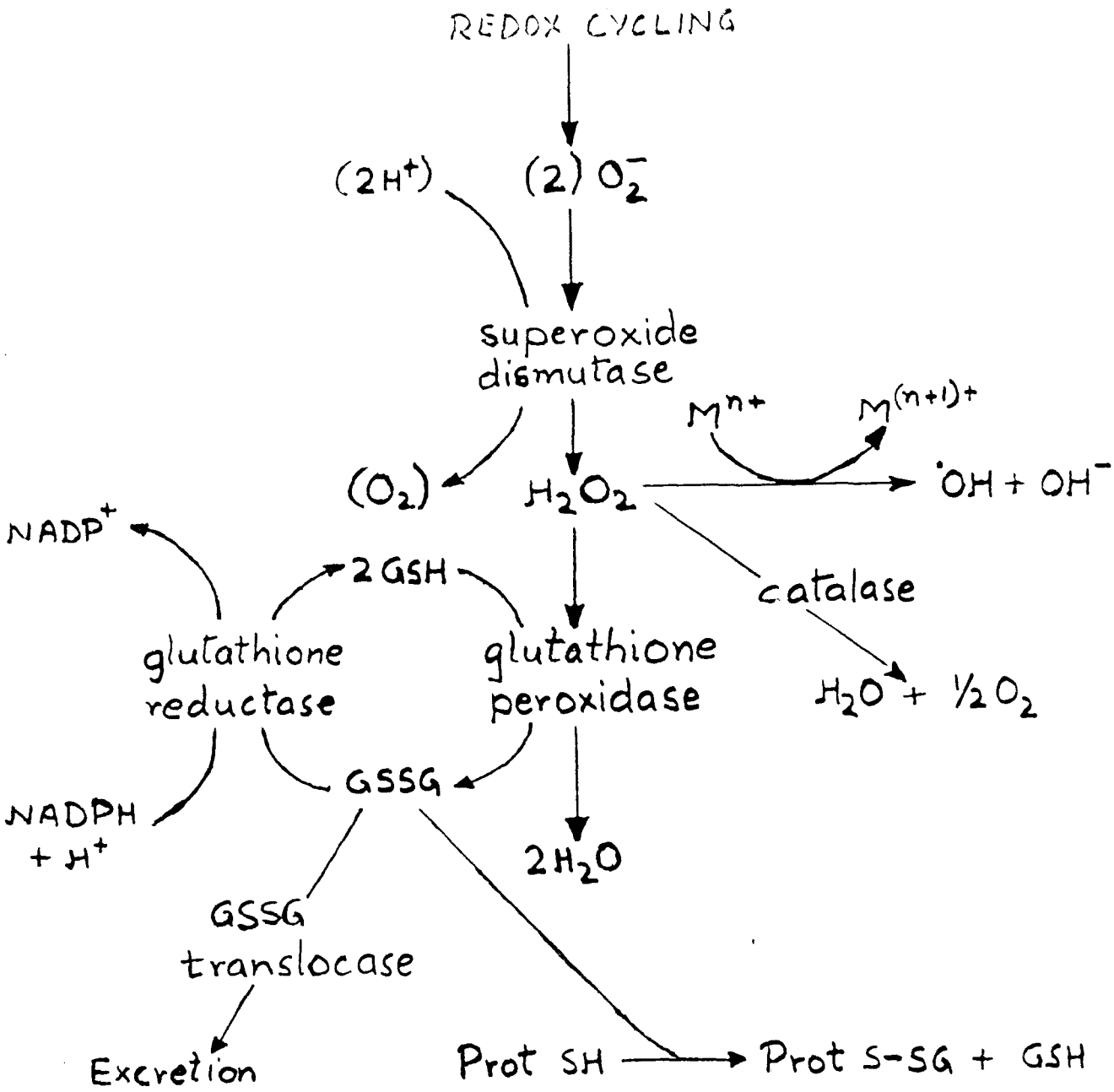


Fig. VI: Thiol-recycling during free radical generation.

In animal studies GSH/GSSG ratios can be decreased acutely *in vivo* by the administration of agents that deplete GSH, but which cause no observable adverse effects (Kehrer and Lund, 1994). However, the effects of a chronic deficit of GSH would subject the individual to a greater probability of not being able to respond to additional stresses and shifts in thiol/disulfide ratios might have some inherent primary effects through shifts in protein thiol/disulfide status (Gilbert, 1989; Ravindranath and Reed, 1990; Nicotera *et al.*, 1992a). Depletion of GSH and production of lipid hydroperoxides has been observed in liver mitochondria after acute ethanol administration in rats (Trenti *et al.*, 1992).

The plasma GSH concentrations of 1 to 10 um are substantially lower than the 1 to 10 mM concentrations found in most cells, but plasma GSH appears to serve important physiological functions, one of which appears to be the delivery of GSH and/or cysteine to extrahepatic tissues. Steady state concentrations of GSH in plasma reflect the dynamic balance between input and clearance. Lauterberg *et al.* (1984) showed that greater than 90% of the input of GSH to the circulation was attributable to efflux from the liver. Recently, roles of glutathione in neutrophil locomotion and neuronal receptor modulation have been described by Elferink and deKoster (1991). Within cells, glutathione is kept in the GSH form by the action of the flavoenzyme, glutathione reductase (GR), which catalyses the reduction of GSSG at the expense of NADPH:

$$NADPH + GSSG + H^+ \quad \underset{<----}{\overset{---->}{}} \quad NADP^+ + 2GSH$$

In erythrocytes, NADPH is regenerated from $NADP^+$ by glucose -6- phosphate dehydrogenase (GPDH) and 6- phosphogluconate dehydrogenase (Becker *et al*, 1995)

High activities of G6PDH and GR, the two functionally linked enzymes might indicate a response to oxidative stress in biological systems (Lopez-Torres, 1993). Oxidative cell injury is usually accompanied by perturbations of thiol homeostasis ie. depletion of soluble thiols, mainly glutathione (GSH) and protein bound thiols. GSH-depleted cells have been widely used as a valuable experimental system to study cytotoxicity (Reed, 1990; Jevtovic-Todorovic and Guenthner,1992).

Thiol oxidation has been related to disturbances of Ca^{2+} homeostasis by several hepatotoxins eg., acetaminophen (Moore *et al* 1985) etc. The underlying mechanisms were supposed to be inactivation of Ca^{2+}- translocating ATPases as well as inhibition of mitochondrial Ca^{2+} sequestration (Kass, 1992), either by modification of specific protein

thiols or by an imbalance of the mitochondrial glutathione redox system. Nahagawa *et al.* (1992) showed that protein thiol oxidation starts early in cellular injury. The changes observed in the regional GSH/GSSG ratios due to cadmium neurotoxicity (Shukla *et al.*, 1988) could be the result of glutathione reductase activity, as this enzyme catalyzes an irreversible conversion of GSSG to GSH which is responsible for higher cellular GSH levels. Therefore,GR is a secondary antioxidant enzyme that helps in the detoxification of active oxygen species by decreasing lipid peroxides or by maintaining the activity of the primary antioxidant enzyme (Sugiyama, 1994). Three mechanisms have been invoked to account for the loss of protein thiol groups (DiMonte, 1984)

(1) Protein thiols are lost as a result of their reaction with GSSG to form glutathione-mixed disulfides.
(2) A loss of protein thiols follows their direct oxidation to disulfides, presumably by partially reduced oxygen species.
(3) In the case of the toxicity of menadione, protein thiols are lost as a result of their arylation by the quinone itself.

In addition to the formation of glutathione mixed disulfides, loss of protein thiols has been shown as a result of the peroxidation of membrane lipids during the intoxication of cultured hepatocytes with the H_2O_2 generated in the cultured medium by glucose oxidases (Kyle *et al*, 1989). However, it is likely that this loss is simply an epiphenomenon of the direct destruction of membrane integrity by the peroxidation of unsaturated fatty acids.

Antioxidant defenses

When ROS are generated in living systems, a wide variety of antioxidants come into play (Sies, 1991). The relative importance of these as protective agents depends on which ROS is generated, how it is generated, where it is generated and what target of damage is measured (Halliwell and Gutteridge, 1995)

Vroegop *et al* (1995) showed in a cell culture system that upto a certain limit, the cells are able to control the damage with glutathione, catalase, SOD, or other antioxidant mechanisms. Once a threshold of damage or rate of damage is exceeded, however, the cellular defenses are overwhelmed and even a slight additional insult results in severe cellular injury. The results led to two important and related conclusions: (a) There is a specificity to the location of damage produced in cells by particular ROS. (b) It is unlikely

that a single protective agent will be able to protect from all the toxic species that may be generated near cells. Especially because some toxic species are likely to be restricted to lipophilic sites and other toxic species to hydrophilic sites, it is prudent to consider developing protective compounds that are specific for each of these types of sites.

In view of the potential adverse effects of oxygen and its reactive intermediates, it is important that a number of antioxidant defense systems be present in the cell. Therefore, all cells have a battery of antioxidant enzymes which are necessary for the survival of the cell even in normal conditions and they act in a cooperative or synergistic way to ensure a global cell protection (Michiels *et al.*, 1994). However, optimal protection is achieved only when an appropriate balance between the activities of these enzymes is maintained. The cell may protect cellular components from the deleterious effects of oxidative stress by:-

(1) direct interaction of oxidants and oxidizing agents with ascorbic acid, reduced glutathione (GSH), and the reducing agents;
(2) scavenging free radicals and singlet oxygen with vitamin E, ascorbic acid, β-carotene and SOD;
(3) reducing hydroperoxides via activities of GSH peroxidases and catalase;
(4) binding or removal of transition metals with various chelators;
(5) separating or preventing ROS from reacting with essential cellular components or from reaching the specific site of action through the membrane barrier;and
(6) replacing or repairing resultant damage via metabolic activities (Chow, 1988; Amstad *et al.*, 1991).

Taking all these factors into consideration, Halliwell and Gutteridge (1995) define an antioxidant as "any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate. This definition emphasizes the importance of the source of stress and the target (oxidisable substrate) measured.

A large number of chemicals and drugs have been shown to be capable of causing direct oxidation of biological compounds generating free radical intermediates and/or altering homeostasis of transition metals eg. paraquat (Ogata and Manabe, 1990), acetaminophen (Rao *et al*,1990) etc. Generation of free radical intermediates appears to be the common mechanism involved in the toxic effects of these compounds.

Variations in the levels of antioxidants leads to differential tissue response to oxidative stress. It has been reported that the rat brain possesses a relatively weak antioxidant defense system (Benzi *et al*,1989). In spite of its high O_2 consumption, the brain curiously contains less active SOD,catalase and peroxidases than other enzymes (Choi and Yu,1995).The involvement of lipids and membranes in essential brain functions has been well established (Aprikyan and Gekchyan, 1988).

Lopez- Torres *et al.* (1993) showed that catalase depletion led to very strong tissue - specific and time -dependent reactive increases of SOD, GR, GSH, and ascorbate. These inductions can explain the very good tolerance to CAT depletion. Induction of endogenous antioxidants has been observed at the gene level in bacteria (Storz *et al*,1990), Neurospora (Munkres,1990),and even in human cells (Rushmore *et al*,1991) in response to H_2O_2 or $O_2^{\cdot-}$.

The induction of SOD,GR,GSH,and ascorbate can help to control OH indirectly (due to $O_2^{\cdot-}$ scavenging by SOD or reduction of GSH back to GSSG by GR) or directly (GSH and ascorbate) even in the presence of high H_2O_2 values. It has thus been proved that a global increase in antioxidant capacity can increase resistance to factors causing early death, leading to an increase in mean life span (Ravindranath *et al.*, 1989). This would be consistent with epidemiologic evidence supporting the concept that levels of endogenous antioxidants correlate with a lower incidence of cardiovascular diseases (Gey,1990) or cancer (Comstock,*et al*,1991).

Aging has also been shown to be related to oxidative damage e.g.,an early death mutant of Drosophila melanogaster exhibited decreased SOD concentration. Long-lived houseflies selected,from a large population, had more SOD,catalase,and GSH and less peroxide than the short -lived members of their cohort(Sohal *et al* 1986). GSH can react with a broad range of radicals ($R^{\cdot}$ from parent compound RH), some at diffusion-controlled rates (VonSonntag and Schuchmann,1990).

$R^{\cdot} + GSH \quad <-----> \quad RH + GS^{\cdot}$ (1)

$GS^{\cdot} + GS^{-} \quad <-----> \quad GSSG^{\cdot-}$ (2)

$GSSG^{\cdot-} + O_2 \quad ------> \quad GSSG + O_2^{\cdot-}$ (3)

Even though reaction (1) is not always thermodynamically favourable, it can be kinetically driven by the removal of the glutathione thiyl radical through (2) and (3) enabling GSH to act as an efficient scavenger. (Winterbourn, 1993). As a consequence, radical scavenging by GSH leads to superoxide and to be effective, requires superoxide

removal through the synergistic action of SOD (Munday and Winterbourn, 1989). Glutathione (gamma- glutamylcysteinylglycine; reduced glutathione, GSH) is a tripeptide found ubiquitously in plant and animal tissues, which functions to protect tissues from the toxic effects of many exogenous and endogenous substances. The cysteinyl thiol group of GSH may conjugate non-enzymatically or bind with electrophilic substances via glutathione-S-transferase (GST) to form a premercapturate compound (Trenga *et al*, 1991). GSH also provides regulatory power to prevent oxidative damage in tissues (Spina and Cohen, 1989). The depletion of intracellular GSH, and subsequent remarkable rebound increase in concentration in response to oxidative stress, may reflect the production of superoxide radicals through redox cycling, with subsequent formation of H_2O_2 and ·OH radicals (Romero *et al.*, 1995). H_2O_2 and peroxide radicals resulting from the combination of lipid and DNA radicals with oxygen may be reduced by GPx in the presence of H^+ donors such as GSH to produce hydroperoxides and the glutathione radical, which rapidly self-associates to form GSSG (Biaglow, 1986). Rat brain mitochondria differ from those in liver in that GSSG levels do not increase following oxidative stress,(Ravindranath,1994) and most of the depleted GSH is recovered as glutathione-protein mixed disulphide with extensive modification of protein thiols including the formation of mixed disulfides of proteins (Pr-SS-Pr). A bulk of evidence has been gathered in recent years showing that peroxidative decomposition of PUFAs and ensuing membrane perturbations always result due to chemical toxicity. It is also being accepted that such changes are the effects of an early unspecific event in toxicity rather than specific causative mechanisms. In most of these cases, various free radical scavengers and anti-oxidants were found to reverse these changes, and induction of SOD was found to be a defense mechanism against oxidative stress (Kakkar and Viswanathan,1990).

The neurotoxic effects of some chemicals may be modulated, at least,in part, by brain GSH levels. Brain levels of GSH are generally quite stable and agents that decrease its concentration in peripheral tissues do not usually alter brain GSH levels. This may be due to the relatively rapid turnover of GSH in these tissues compared to that in brain (Griffith and Meister, 1979). In addition, the blood-brain barrier apparently protects the brain from many of the insults that lead to GSH depletion in other organs. (Trenga *et al*, 1991).

The significance of GSH depletion seems to be that certain critical protein thiols become oxidized and Ca^{2+} pumps inactivated. Calcium levels in the cytosol then increase with catastrophic consequences. (Cheeseman, 1993). $O_2^{\cdot-}$ generated reacts with GSH to cause an increase in O_2 consumption, and GSSG formation, both of which are fully inhibited by SOD. Winterbourn and Metodiewa (1994) showed in their study with the xanthine oxidase/ hypoxanthine system that little, if any, of the additional O_2 consumed is converted to H_2O_2. They further showed that approximately 90% of the GSH is oxidized to GSSG, the remainder being converted to sulfonic acid. The efficiency of the reaction increased with increasing GSH concentration (1-8 mM), pH and pO_2 and with decreasing $O_2^{\cdot-}$ generation rate.

Thus, the results show a superoxide-dependent chain that does not produce H_2O_2 and that is terminated primarily by superoxide dismutation. This may occur via an initial reaction of $O_2^{\cdot-}$ with GSH giving rise to sulfinyl radical rather than transfer to give thiyl radical. Thus, GSH at the millimolar concentrations found intracellularly should react with $O_2^{\cdot-}$, but because O_2 is regenerated it will not be an effective scavenger. Physiologically, SOD is required to prevent chain oxidation of GSH.

Enzymatic antioxidant defenses include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) etc. Alongwith these many more enzymes act as antioxidants like glucose-6-phosphate dehdyrogenase, which are involved indirectly and are thus known as secondary antioxidants.

Superoxide dismutase (SOD)

SOD exists in two forms in eukaryotes: copper-zinc superoxide dismutase (Cu-Zn SOD) which is found primarily in the cytosol of cells, and manganese superoxide dismutase (Mn SOD) which is located mainly in the mitochondria. This enzyme is involved in facilitating the dismutation of the superoxide free radical to a non-free radical species (H_2O_2) (Fridovich, 1983). A number of agents which cause increased oxygen radical production in cells, such as paraquat, bleomycin and molecular O_2 have been shown to cause an increase in SOD activity.

The enzymatic activity of SOD was discovered by its ability to inhibit the reduction of cytochrome c by xanthine oxidase. It catalyses the dismutation of $O_2^{\cdot-}$ substrates near

the diffusion control limit (Kcat=$2x10^9$ M^{-1}. S^{-1}, Fridovich, 1989), about 10^4 times faster than spontaneous dismutation

$2O_2^{\cdot-} + 2H^+$ ------> $O_2 + H_2O_2$

(McCord and Fridovich, 1968).

$O_2^{\cdot-} + H^+$ ----------> $HO_2^{\cdot}$

$HO_2^{\cdot} + O_2^{\cdot-} + H^+$ ----------> $H_2O_2 + O_2$

---

net reaction: $O_2^{\cdot-} + O_2^{\cdot-} + 2H^+$ -------> $H_2O_2 + O_2$

Ubiquity of SOD in all aerobic organisms was taken to imply that the $O_2^{\cdot-}$ radical is universally produced by aerobic metabolism,
and also that it is sufficiently toxic to require the presence of a detoxification system (Mc cord *et al*, 1971; Kakkar, 1985).

(a) Cu-Zn SOD

The Cu-Zn SODs are, in general, found in the cytoplasm of eukaryotic organisms. They are dimeric molecules. Each of the identical subunit contains one atom of copper and one atom of zinc, which are tightly but reversibly bound to a polypeptide chain of 16,000 MW (Richardson, 1977). Only the copper ions are catalytically active, undergoing cyclic reduction and oxidation as follows:

Enz-Cu^{++} + $O_2^{\cdot-}$ -------> Enz-Cu^{+} O_2

Enz-Cu^{+} O_2 + $2H^+$ ------> Enz-Cu^{++} + H_2O_2 (Fee, 1977).

Role of Cu-Zn SOD has been studied in a number of pathological conditions and mutations in Cu-Zn SOD gene has been shown to be associated with familial amyotrophic lateral scelrosis, a chronic neurodegenerative condition (Rosen *et al.*, 1993).

(b) Mn and Fe SODs:

Though these possess the same enzymatic activity as the Cu- Zn SOD, yet they are clearly different being larger in size (40,000 MW) and lacking the unusual stability of the Cu-Zn SODs. All Mn and Fe dismutases show a high degree of homology but the

copper-zinc enzyme appears to be evolutionarily unrelated (Harris and Steinman, 1977). Both the Mn and Fe SODs are present in the matrix space of mitochondria (Britton and Fridovich, 1977). Even though the iron and manganese enzymes are closely related, and in both cases, the metal ions may be reversibly removed from the proteins, only the original ion is capable of restoring activity to the apoproteins (Mc cord, 1979). Under conditions of oxidative stress, whether caused by hyperoxia or by various toxic agents which increase $O_2^{\cdot-}$ production at normal O_2 concentration, organisms appear to respond by increasing the amount of SOD in their cells.

In a variety of mammalian cells and tissues, MnSOD is a highly inducible enzyme responding to a variety of stimuli, including H_2O_2 in some cell types. In contrast, Cu-Zn SOD appears to be constitutively expressed at higher levels and is considerably less inducible than Mn SOD in mammals (Visner *et al*, 1990). White *et al* (1993) demonstrated that the expression of MnSOD in vivo was not affected by overexpression of the CuZn SOD and , therefore, the two enzymes are probably regulated independently. Furthermore, Nimrod *et al* (1994) have shown that Mn-SOD is superior in most of the model systems. This property can be attributed to its longer biological half-life.

There has been a debate in the literature concerning the mechanism of SOD-mediated protection against tissue injury. Different authors have suggested that SOD is protective because a lower steady-state concentration of O_2- would result in less ·OH through the iron catalyzed O_2 driven Fenton reaction (Halliwell and Gutteridge, 1986). Although this is possible, the reaction of $O_2^{\cdot-}$ with Fe^{3+} is relatively slow ($k=1x10^6 M^{-1}. S^{-1}$) and reductants such as ascorbate and glutathione, that can exist at much higher concentrations than $O_2^{\cdot-}$ (mM vs nM range) can substitute for $O_2^{\cdot-}$ in the Haber-weiss cycle (Radi, 1993). Thus, most of the $O_2^{\cdot-}$- mediated toxicity may be due to its direct reactions with critical biomolecular targets. $O_2^{\cdot-}$ is not a very reactive free radical (Sawyer and Valentine, 1981) but can still be toxic for some specific targets because it can diffuse across great enough distance to attack a critical residue before disappearing by means of reactions with non-critical ones. eg. ironsulfur clusters in proteins are oxidised by $O_2^{\cdot-}$ and SOD protects these by scavenging O_2 at almost diffusion controlled rates.

Diminution or elimination of SOD will increase sensitivity of cells to oxygen and to compounds which can divert univalent e^- flow to oxygen; however, elevation of SOD

above wild type levels should therefore, not be expected to provide complete protection against overproduction of $O_2^{\cdot}$ (Fridovich, 1993). SOD also exerts part of its antioxidant protection by inhibiting the formation of the cytotoxic $ONOO^-$. The dismutation product of $O_2^{\cdot}$, H_2O_2, is less reactive than $ONOO^-$ and can be metabolized by specific enzymatic systems.

Steinman and Weinstein (1993) reported that Mn-SOD is more effective than Fe-SOD in preventing oxyradical damage to DNA in vivo and have further suggested that localization near targets of oxyradical stress may enhance the effectiveness of SOD in preventing oxyradical damage to those targets. This suggestion has important implications in engineering SODS for clinical applications. Thay have also shown that purified E. coli MnSOD binds DNA which demonstrates that SODs may have biological "activities" other than superoxide dismutation.

During action of oxidants on SOD, its structure is strongly changed and new molecular forms appear which possess the catalytic activity. -SH groups of SOD are easily oxidized during the action of oxidants but its oxidation does not accompany the loss of functional activity. Thus, in contrast to oxidation of NH- groups, oxidation of -SH groups have the positive effect because -SH groups actively react with oxidants and play the role of "false" target for oxidants. (Sharonov and Churilova, 1993).

Catalase

It is a ubiquitous enzyme found in all organisms. In eukaryotes, it is largely sequestered in the peroxisomes (Halliwell and Gutterdige, 1989) and may have some significance in the general intracellular scavenging of hydrogen peroxide by disproportionation.

$$2H_2O_2 \text{ ----------> } 2H_2O + O_2$$

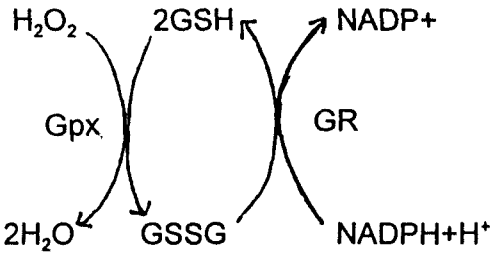
This enzyme contains a heme in its active site, which is responsible for its catalytic activity. This is an intracellular enzyme because cells contain iron and probably copper ions in forms that can accelerate damaging free radical reactions. Thus, catalase helps in preventing the accumulation of H_2O_2 within cells.

The brain, heart and skeletal muscles contain only low amounts of catalase, although the actvity does vary between muscles and even among different regions of the same muscle (Marklund *et al.*, 1982). Thus, H_2O_2 produced by glycollate oxidase and urate oxidase in the peroxisomes is largely removed by catalase whereas that

produced by soluble cytosolic enzymes such as SOD is acted upon by GSH-Px which is found mainly in the cytosol and in the matrix of mitochondria (Halliwell and Gutteridge, 1989).

Glutathione Peroxidase (GPx) and Glutathione Reductase (GR)

Glutathione peroxidase (GPx) decomposes H_2O_2 to H_2O at the expense of reducing equivalents from GSH. GPx is located in both the cytosolic and mitochondrial compartments. Once GSH is oxidized to GSSG, GSSG is reduced back by the action of NADPH- dependent glutathione reductase.



Normally, most intracellular glutathione is in its reduced form. Increased intra cellular concentrations of H_2O_2 result in a drop of the GSH/GSSG ratio, which serves to detect intracellular oxidative stress (Tribble and Jones, 1990). GPx appears to be the most important H_2O_2 detoxifying system under physiological conditions, due to its low Km for H_2O_2 (Chance *et al*, 1979)

Like SOD and other oxidatively denatured proteins, GPx inactivated by peroxides or free radicals seems to be preferentially degraded by proteases (Pigeolet and Remacle, 1991) Glutathione Reductase is a dimeric enzyme containing FAD in its active siteS, which utilizes NADPH to reduce GSSG. The ratios of GSH/GSSG in normal cells are generally high, being > 10/1 in rat brain (Halliwell and Gutteridge, 1989). To achieve this, the GSSG must be reduced back to GSH, a reaction catalyzed by GR:

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

The activity of GR in rat brain is found to be moderate as compared to that in the liver where it is quite high (Halliwell and Gutteridge, 1989). The NADPH required for this reaction is provided in animal tissues by the oxidative pentose phosphate pathway.

As glutathione reductase operates to lower the NADPH/$NADP^+$ ratio, the pentose-phosphate pathway speeds up to replace the NADPH.

Apparently, during the catalytic cycle the NADPH reduces the FAD at active site of the enzyme which then passes its electrons on to a disulfide (-S-S-) between two cysteine residues in the protein. The two -SH groups so formed then interact with GSSG and reduce it to 2GSH, thereby reforming the protein disulfide (Desphande *et al.*, 1996).

Non-enzymatic Antioxidants

Some of the important non-enzymatic antioxidants, besides thiol are beta-carotene, ascorbic acid, alpha-tocopherol and GSH apart from the synthetic antioxidants which are increasingly being used in food preservation, cosmetics, drugs and other industries.

β-carotene

It has been identified as a sacrificial inhibitor of the propogation stage of lipid peroxidation(LPO) as it is a quencher of singlet oxygen. Thus, it prevents formation of hydroperoxide and intereferes with LPO, but at higher oxygen pressures, beta- carotele may become prooxidant (Burton and Ingold, 1984).

Vitamin C

It is hydrophilic in nature and is primarily found in the cytosol and functions directly as an antioxidant and is an essential entity for the proper functioning of the various cellular defense mechanisms. It also appears to be involved in the biotransformation of xenobiotics and influences the activity of several oxidizing and hydroxylating enzymes (Davies *et al.*, 1991). Its antioxidant reactions use its ability to donate a single electron to the free-radical species. The products of such reactions are the quenched reactive spcies and the less reactive ascorbyl free radical, which can be either reduced back to ascorbic acid or oxidized to form dehydroascorbic acid (Bendich *et al.*, 1986).

(a) ascorbate + Fe^{3+}-complex ----------> Fe^{2+}-complex + semidehydro ascorbate

(b) 2-semidehydroascorbate ----------> ascorbate + dehydroascorbate

(c) dehydroascorbate + 2GSH ----------> GSSG + ascorbate

dehydroascorbate reductase

complex reaction

oxalic acid + L-Threonic acid

Ascorbate reacts rapidly with both superoxide and peroxyl radicals and even more rapidly with hydroxyl radicals (Halliwell and Gutteridge, 1989). It also scavenges singlet oxygen and reduces thiyl radicals and protects tissues against LPO (Chakraborty *et al.*, 1994). It plays a vital role in maintaining the balance between oxidative products and the various cellular antioxidant defense mechanisms. It is required as a cofactor for some enzymes such as proline hydroxylase, dopamine beta- hydroxylase etc. At low concentration it can act as a prooxidant in the presence of H_2O_2. Human eye lens has low SOD activity but high content of ascorbic acid.

α-tocopherol

It is the principal component of the secondary defense mechanism against free-radical mediated cellular injuries. In fact, it is the only natural physiological lipid-soluble antioxidant that can inhibit lipid peroxidation in cell membranes (Kappus, 1991). Evidence includes such *in vitro*, observations as its direct reactions with and quenching of superoxide and peroxyl radical and singlet oxygen.

Vit.E-OH + $ROO^{\cdot}$ --------> Vit. E-$O^{\cdot}$ + ROOH (H transfer)

Vit.E-OH + $ROO^{\cdot}$ ---------> Vit. E-$OH^{\cdot+}$ + ROO^{-} (e^{-} transfer)

Vit.E-$OH^{\cdot+}$ + H_2O ---------> Vit. E-$O^{\cdot}$ + H_3O^{+} (deprotonation)

It is now widely recognized that tocopherol is located primarily in the membrane portion of the cell and is a part of the cells defense against oxygen centered radicals. Alpha-tocopherol can function as a molecular "channel" via which the free raidcals can leave the hydrocarbon zone of the membrane. Almost all enzymes that are affected by vitamin E status either are membrane bound or are concerned with the GSH-Px system (Catigani, 1980). Vitamin E, therefore, is unique in its more specific localization in membranes and the tenacity with which it remains in most tissues. It is widely used clinically and is capable of preventing haemolysis and retrolental fibroplasia in infants.

The autooxidation and antioxidant reactions involving vIt. E are summarized as follows:

1. Initiation (formation of a free radical)

$$LH \xrightarrow{\text{Initiators}} L\cdot$$

2. Reaction of radical with oxygen

$$L\cdot + O_2 \longrightarrow LO_2\cdot$$

3. Propagation

$$LO_2\cdot + LH \longrightarrow L\cdot + LOOH$$

4. Antioxidant reaction

$$LO_2\cdot + E \longrightarrow E\cdot + LOOH$$

5. Regeneration

$$E\cdot + C \longrightarrow E + C\cdot$$

$$C\cdot + NADPH \xrightarrow[\text{ascorbate reductase}]{\text{semidehydro}} C + NADP$$

$$E\cdot + 2GSH \longrightarrow E + GSSG$$

$$GSSG + NADPH \xrightarrow{\text{Gred}} 2GSH + NADP$$

6. Termination

$$E\cdot + E\cdot \longrightarrow E - E \text{ (dimer)}$$

(L. = fatty acid radical; $LO_2\cdot$ = peroxyradical, LH = fatty acid; E = tocopherol; LOOH = hydroperoxide; C = Ascorbic acid; C· = Ascorbyl radical)

Thus, there exists an interdependency of the various cellular antioxidant defense mechanisms involving ascorbic acid, vitamin E, selenium, catalase and GSH.

Apart from the main non-enzymatic antioxidants mentioned above, some more cellular components have been identified which can act as antioxidants. These include **Uric acid** which is present in human blood plasma at concentration of 0.12 - 0.45 mM/l where activity of SOD and catalase is comparatively low. It is a powerful scavenger of $^1O_2$, and ·OH. As compared to ascorbic acid it is more effective in trapping proxyl

radicals in the aqueous phase. **Zinc** has also gained importance as an antioxidant due to its capacity to displace transition metals from site of action and prevent Fenton and Haber-Weiss reaction. However, its role as a dietary antioxidant has been debated by Samman (1993). Zn in high doses (45 mg/100 g diet) acts as antioxidant probably by induction of metallothionein but it also hampers the intestinal uptake of Cu, thus leading to reduced SOD activity.

Albumincan loosely bind to Fe^{2+} and Cu^{2+} and prevent Fenton's reaction. Its amino acids like histidine, tryptophan and methionine can scavenge $O_2^{\cdot}$ and OH radicals. **Estrogen** has been shown to scavenge radicals and suppress lipid peroxidation whereas **Bile pigments** are potent scavengers of $O_2^{\cdot}$ and HOCl in a synergistic action with Vit. E. It can also scavenge peroxyl radicals with high efficiency. Thus any metal or chelating agent capable of removing transition metal ions from the site of action (Morel *et al.*, 1992) may prove to be an antioxidant.

LIPID PEROXIDATION: A Consequence of Free Radical Generation

The mammalian brain shows an important rate of oxidative metabolism resulting in a high consumption of molecular oxygen, but contains relatively low levels of antioxidants (Harmann, 1992). It is protected from chemical insult by the blood-brain barrier (BBB) which prevents the entry of most polar xenobiotics in the brain. (Pardridge, 1983) Therefore, only lipophillic xenobiotics can cross the BBB and reach the brain where they may be metabolized by several enzymatic systems (Minn *et al*,1991).

Lipid peroxidation (LPO), is probably the most extensively studied of free radical related processes in biological context. LPO can be defined as the oxidative destruction of lipids and in most cases refers to fatty acid oxidation (Cheeseman,1993). The significance of LPO as a damaging process is that (i) its most susceptible targets are the polyunsaturated fatty acids (PUFA) that are common in all cell membranes and (ii) once initiated, it is a self perpetuating chain reaction.

The general process of LPO can be envisioned as in Fig. VII where LH is the target PUFA, R·, the initiating radical, L· the carbon-centred fatty acid radical, LO·, a fatty acid alkoxyl radical, LOO· a fatty acid peroxyl radical and AH any hydrogen donor:

LH+R· ----------> L·+RH (hydrogen abstraction)

L·+O_2 ----------> LOO· (oxygen addition)

LOO·+LH ----------> LOOH+L·etc.

LOO·+AH ----------> A·+LOOH

Hydrogen abstraction is invariably from a bis-allylic methylene group destabilised by adjacent double bonds. The resultant carbon-centred fatty acid radical (L·) immediately rearranges forming conjugated diene structures. These rapidly add oxygen to form lipid peroxyl radicals (LOO·) that can in turn abstract hydrogen from a suitable source such as AH in the scheme above. AH may be an antioxidant molecule: if A· is unreactive, it will effectively terminate the peroxidation process. If the LOO· radical abstracts hydrogen from another PUFA, it will begin another radical chain. Hence, lipid peroxidation is a process initiated and propagated by free radicals resulting in destruction of the unsaturated fatty acids of membrane phospholipids (Farber, 1994).

The breakdown of lipid hydroperoxides is a scission reaction that always generates another free radical and an aldehydic product. Cell membranes represent a mixture of PUFA, mostly linoleic, arachidonic and docosahexaenoic acids containing 2, 4 and 6 double bonds, respectively. Their respective primary fatty acyl radicals can rearrange to a number of different isomers yielding peroxide products from the fatty acids. Since each lipid hydroperoxide can undergo two different scission reactions, the number of different secondary fatty acyl radicals and aldehyde products is very large. Moreover, some of the aldehyde products of membrane PUFA production will be released while others remain attached to the parent phospholipid. In short, lipid peroxidation of biological membranes yields a complex pattern of aldehyde products. Esterbauer and co-workers have been foremost in characterizing the aldehyde products of biological LPO (Esterbauer *et al.*, 1991).

Halliwell and Chirico (1993) observed that "the detection and measurement of LPO is the evidence most frequently cited to support the involvement of free radical reactions in toxicology and human disease". This emphasis is because of the following reasons.

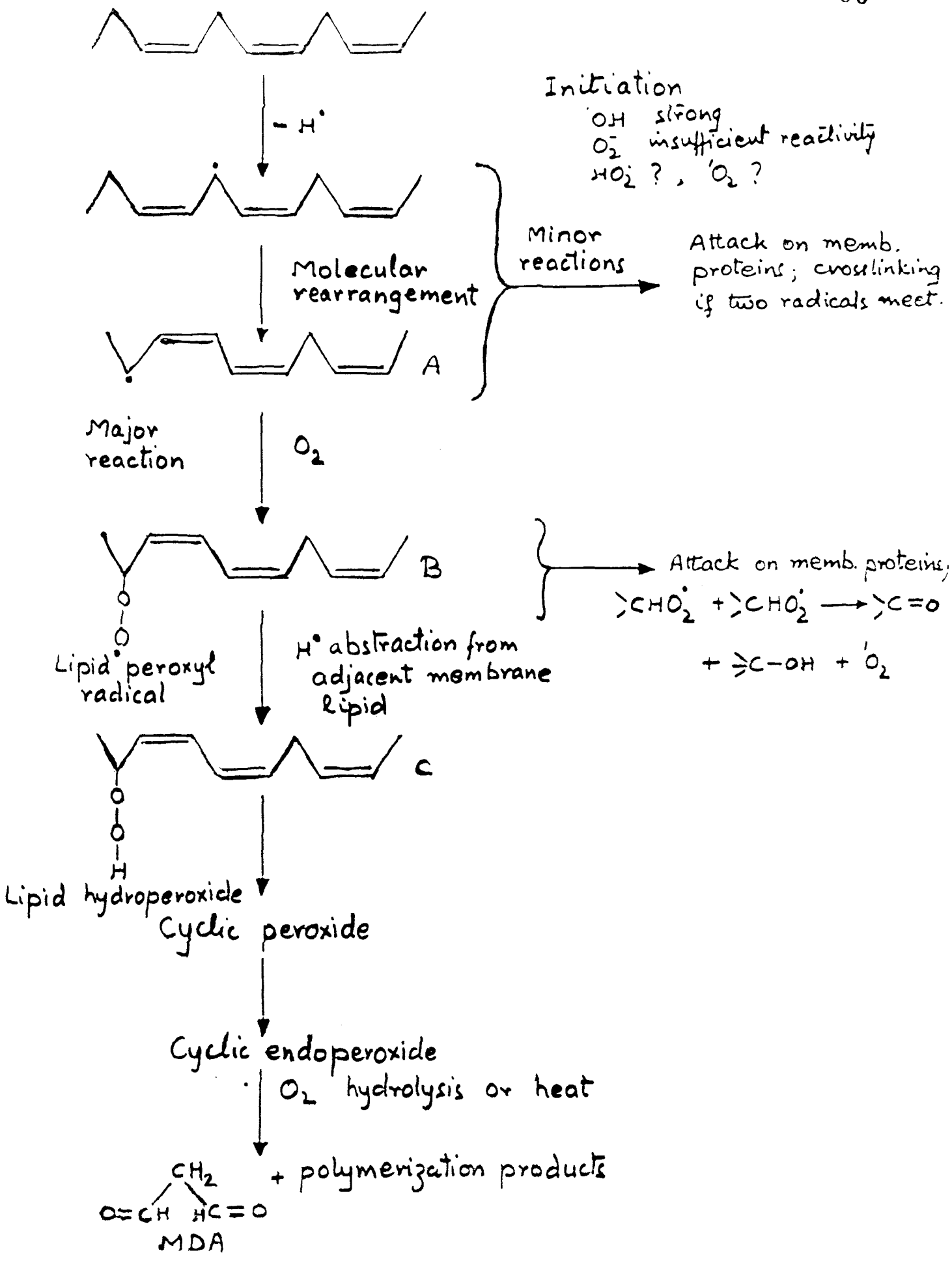


Fig. VII: General process of lipid peroxidation.

1. It is one of the most likely consequences of reactive free radical production in a cell since the PUFA substrates are so abundant.
2. It is a very destructive process, being a chain reaction in nature and attacking the membrane structures that are essential for normal cell metabolism and viability.
3. A vast amount of expertise has been developed to study LPO more so than has hitherto existed to study other processes.

The extreme damaging potential of LPO makes it one of the most important processes in free radical biochemistry. It is damaging directly through its effects on the cell membranes and indirectly by the production of aldehydes. The direct effects on the cell membranes include changes in the biophysical properties of the membrane: fluidity is decreased due to loss of PUFA, electrical resistance decreases, protein mobility in the membrane is decreased and phospholipid exchange between halves of the bilayer is facilitated (Richter *et al.*, 1987). Destruction of membranes leads to the inactivation of membrane bound enzymes and the loss of decompartmentalisation that is essential for ordered cell metabolism. Clearly, uncontrolled LPO could represent a catastrophic occurrence for a cell.

The indirect effects of LPO are more subtle but may be also extremely important as LPO of cell membranes yields a complex variety of aldehydes and some of these have potent biological activity. The main classes of aldehyde products are the alkanals, alkenals and hydroxyalkenals. Of these, the most biologically active are the hydroxyalkenals and the most important member of that class is 4-hydroxynonenal (HNE), which is both a major aldehyde product and has potent biological activity. HNE reacts with thiols (including GSH and protein - SH), inhibits a wide range of enzyme processes including synthesis of DNA, RNA and protein, mitochondrial respiration, calcium pump activity of the endoplasmic reticulum and cell division (Schaur *et al.*, 1991).

Of all the effects of LPO, possibly the ultimate lethal event is the loss of calcium homeostasis due to destruction of membranes and inactivation of ion pumps. Loss of ability to pump calcium ions out of the cytosol has been found to be a lethal event possibly common to a number of cytotoxic processes (Kakkar *et al.*, 1995). A sustained increase in cytosolic Ca^{2+} can activate proteases, phospholipases and endonucleases causing DNA fragmentation, disrupted cell signalling and distortion of the cytoskeleton (Orrenius *et al.*, 1989).

LPO is one of the best known manifestations of oxidative cell injury.It has been established in one model of such an injury, namely the iron-dependant killing of cultured hepatocytes by t-BHP, that the lethal cell injury is, in fact, mediated by the peroxidation of membrane lipids (Masaki *et al.*, 1989). Fauconneau *et al.* (1994) showing that TBARS production and Fe^{2+} release were related to the quantity of total iron present in the brain region. Slices from brain regions provide a good model for LPO study. Since involvement of lipid peroxidation has been seen in a number of toxic manifestations and pathological conditions, the hot topic of debate in recent years has been whether it is the "cause or effect" of toxicity. However, the perturbations in the membrane fluidity (Chen and Yu, 1994) and impermeability due to peroxidative deomposition of polyunsaturated fatty acids does cause disturbances in ionic balance of cells especially Ca^{2+} leading to triggering of many degradative phenomenon and ultimately cell death.

Prooxidative Processes and Ca^{2+} Deregulation in Cell Death

All cells contain eleborate systems for the spatial and temporal regulation of calcium ions, diverse calcium receptors and biochemical response systems that are regulated by these changes in intracellular calcium. Toxicants that perturb the mobilization or homeostasis of Ca^{2+} will place the regulation of these processes outside the normal range of physiological control. Many toxicants including metals, solvents and pesticides may target the:

(a) calcium mobilization and homeostatic processes
(b) calcium mediated processes; and
(c) those processes which co-regulate or counter-regulate these calcium-mediated processes.

A series of reports in the recent past have brought in the concept of a relation between oxidative membrane damage and Ca^{2+} function. Under steady state conditions, the cytosolic free calcium concentration is generally maintained at approximately 10^{-7} M by active efflux i.e. Ca^{2+} pumping ATPase or Na^{+}-Ca^{2+} exchange (anti-port) processes and passive influx (Ca^{2+}-specific channels or pores) of Ca^{2+} across the plasma membrane (Nicotera *et al.*, 1992). Cellular Ca^{2+} is also controlled and modulated by mechanisms other than transport across the plasma membrane. These include high affinity binding by membranes and macromolecules in addition to transport by intracellular organelles. Thus any damage to plasma membrane disrupting the

maintenance of disequilibrium between the internal and external environments may ultimately result in the death of a cell. Recent evidence suggests that mitochondrial damage is the biochemical basis of the non peroxidative mechanism of oxidative cell injury (Masaki *et al*, 1989). That the loss of mitochondrial function can lead to the development of irreversible cell injury is of course, attested to by the effects of ischemia on cells. In this situation, cell killing is correlated with a loss of mitochondrial energization rather than with the depletion of ATP alone (Snyder *et al* 1993). Starke *et al.* (1986) showed that iron- dependent loss of mitochondrial energisation occurred in the absence of lipid peroxidation. Thus, it is very likely that the nonperoxidative mechanism whereby cells are irreversibly injured by activated oxygen species is similar to the mechanism whereby they are injured in the absence of oxygen i.e. by anoxia (Farber, 1994)

Mitochondria act as safety devices against toxic increases of cytosolic Ca^{2+} and also as a major source of intracellular active oxygen species generation. It has low affinity but high capacity for Ca^{2+} accumulation as compared to endoplasmic reticulum which has high affinity but low capacity. Ca^{2+} enters mitochondria via simple facilitated diffusion in response to the large negative inside membrane potential (< 105 mV) generated electron transport dependent H^+ extrusion. Once Ca^{2+} is within the matrix space, it is efficiently buffered to a level of < 0.1% of its total concentration by reversible binding and precipitation. Though, the massive loading of calcium as calcium phosphate disturbs mitochondrial function only negligibly, influx of Ca^{2+} across a damaged plasma membrane is an early common pathway in which cells are killed. Fig. VIII summarises oxidative stress mediated calcium deregulation in apoptotic and necrotic cell death. Ca^{2+} fluxes are important events in cell death and have a role in the phospholipase action exposing polyunsaturated fatty acids to peroxidation. The activation of phospholipase A_2 and the ensuing release of arachidonic acid could trigger the lipoxygenase and cycloxygenase (Fawthrop *et al.*, 1991) cascades and the modulatory influences of the products. Similarly, through diacyl glycerol, phosphoinositide messengers and cyclic ADP-ribose, (Lino and Endo, 1992) further modulation in physiological functions is possible as a consequence of altered calcium status.

The effect of oxidants on mitochondrial structure and function seem to be due to the hydroperoxide-mediated Ca^{2+} efflux which in turn, could be due to the formation of ADP-ribose from oxidised niacinamide co-enzymes, which combine with proteins and

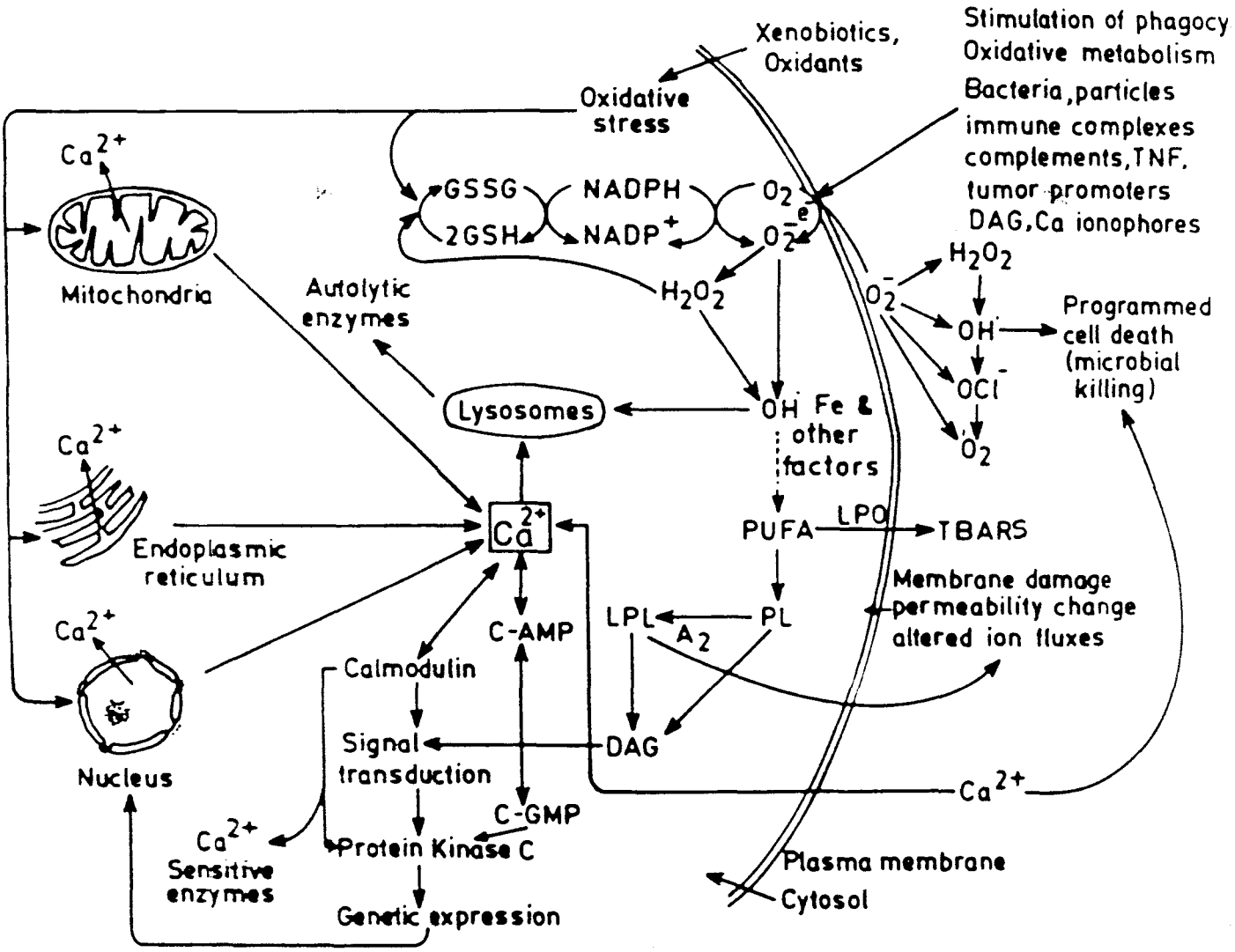


Fig. VIII: Oxidative stress-mediated calcium deregulation in toxicity (Kakkar *et al.*, 1995) (PL-phospholipid; LPL-Lysophospholipid; DAG-Diacylglycerol).

regulate Ca^{2+} fluxes (Richter and Frei, 1988). Sustained increase in intracellular Ca^{2+} concentration can lead to the activation of degradative enzymes like proteinases, phospholipases and endonucleases, ultimately leading to breakdown of macromolecules (Davies and Chipman, 1994). On the other hand peroxidative decomposition of PUFAs and the ensuing structural and functional alterations in plasma membranes and organelles ultimately lead to metabolic changes caused by altered substrate and co-factor accessibility, functional changes in enzymes, undesirable fluxes of cations, and thus biochemical lesions.

In view of the above concepts studies were undertaken to see the effect, if any, of diazepam on free radical generation and antioxidant defenses in brain and its relation to Ca^{2+} responsive events. The results of the initial phase are presented herewith.

GENESIS AND OBJECTIVES

Formation of free radical species during the biochemical response to a wide variety of chemicals and the resultant oxidative stress have been well studied in the past two decades as presented in the Review of Literature. In the recent past, two mechanisms mainly oxidative stress caused by free radicals and destablization of calcium homeostasis have been observed in many cases (Corcoran *et al*, 1994; Kehrer, 1994). These mechanisms gain much more importance in brain tissue as it is quite vulnerable to free radical attack due to its high content of polyunsaturated fatty acids and aerobic metabolism. Peroxidative decomposition of polyunsaturated fatty acids has been associated with several physiological, pathophysiological and toxic processes including ageing in various organs (Warner, 1994). Reactive oxygen species can disrupt cellular calcium homeostasis by inactivating regulatory mechanisms. The resulting loss of control of the asymmetric movement of Ca^{2+} across the plasma membrane can precipitate cell death by activation of potentially destructive biochemical pathways involving phospholipase A_2, neutral protease and lysosomal acid hydrolases.

Nerve cells may be particularly susceptible to reactive oxygen species mediated damage. The differences found in the cellular distribution of GSH and glutathione-S-transferases may contribute to changes in the vulnerability of the nervous system to neurotoxicants at different ages (Lowndes *et al.*, 1994). Brain exhibits tremendous regional heterogeneity and each region displays specialized and selective functions (Ravindranath *et al.*, 1995). The mammalian brain contains relatively low levels of antioxidants (Harmann, 1992) but is protected from blood-borne chemical injury by the blood-brain barrier and the blood CSF barrier which prevent the entry of most polar xenobiotics in the brain. Only lypophilic xenobiotics can cross this barrier and reach the brain. Accumulation of iron in high concentration in brain also makes this tissue at particular risk from free radicals damage.

The formation of superoxide anion and other free radicals alongwith the peroxidative decomposition of polyunsaturated fatty acids of membranes in different regions of brain under neurotoxicity of phenobarbitone., ether and aniline and the adaptive modulation of antoxidant defenses has been reported earlier by this laboratory. The logical explaination of these observation is being pursued. To explore the involvement of such mechanisms during administration of diazepam, a widely used drug is the objective of the present study.

MATERIALS AND METHODS

Animals

Male albino Wistar rats weighing between 150 -200 g, from ITRC Animal Colony were used throughout the study. The animals were maintained on standard pellet diet (M/S Lipton, Bombay, India) and water ad libitum.

Chemicals

Thiobarbituric acid (TBA), phenazine methosulfate (PMS), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide reduced (NADH), 5,5' dithionitro bis 2-nitrobenzoic acid (DTNB) and bovine serum albumin were procured from Sigma Chemicals Co. (St.Louis, MO, USA). Injectible diazepam (Calmpose) was a product from Ranbaxy Laboratories Ltd., India. Sodium pyrophosphate, methanol, trichloro acetic acid and other chemicals were either BDH Analar or E. Merck extrapure.

Animal treatment with diazepam

Diazepam, 3mg/kg, was administered to a group of six animals intra peritoneally and the animals were sacrificed after one hour (Group I).Another set of six animals which were given i.p. saline served as controls. In a separate set of experiments same

number of animals were given diazepam (3 mg/kg) but killed after 18 hours (Group II). This dose which is 1% of the reported LD_{50} dose was chosen after pilot experiments. Experiments were also done with lower (1.5mg/kg) and higher (6.0 mg/kg) doses to observe the relative changes. Schedule for sacrificing the animals remained the same as mentioned above.

Rats were killed by cutting the jugular vein, brain was immediately taken out, rinsed, dried and cleared of all adhering material. Gross dissection of brain regions was carried out quickly according to Glowinski and Iverson (1966). Cerebral cortex, cerebellum and brain stem were removed, weighed separately and a 10% (w/v) homogenate of each portion of brain was prepared in 0.15 M buffered KCl using a Potter Elvehjem glass homogeniser. All the operations were carried out at 0-4 °C temperature.

Preparation of subcellular fractions

Subcellular fractionation of homogenates was done by the method of Mustafa (1974). Nuclear fraction was removed by centrifuging the 10% (w/v) homogenate in 0.15 M buffered KCl, at 2500 x g for 10 minutes in a refrigerated centrifuge. Supernatants were removed and centrifuged again at 12000 x g for 20 minutes to separate mitochondria. Mitochondrial pellets were washed twice with KCl, centrifuged and suspended in distilled water for further processing. Post-mitochondrial supernatant mixed with the first washing was also used for further assays.

Lipid peroxidation

Malonaldehyde formed as a product of lipid peroxidation was estimated at different time intervals 0, 30, 60, and 120 minutes in the homogenate of treated & control animals using the method of Ohkawa *in vivo* (1979). Thiobarbituric acid reactive substance (TBARS) formation was also estimated in mitochondrial and post mitochondrial fractions of cerebral cortex, cerebellum, and brain stem of control and exposed animals.

The reaction system contained 2.0 ml of the 10% (w/v) homogenate, suspended mitochondria or post-mitochondrial fraction and incubated at 37 °C with constant

shaking. Allquots were withdrawn for the assay of TBA-reactive substance formed at 0 time, 30 min, 60 and 120 mins. The optical density was measured at 535 nm. A molecular extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used to calculate nanomoles of malondialdehyde formed per mg of protein per hour.

Measurement of superoxide dismutase activity

SOD activity was determined spectrophotometrically by the inhibition of the formation of formazen in the nicotinamide adenine dinucleotide (reduced)- phenazine methosulphate- nitroblue tetrazolium reaction system as described by Nishikimi et al (1972) and adapted by Kakkar *in vivo* (1984). Cu-Zn SOD was assayed in the PMF and Mn-SOD in the mitochondrial fractions of Group I & II animals. One unit of the enzyme is equivalent to 50% inhibition in the formazan formation in 1 min at room temperature (25 ±2 °C).

Measurement of -SH content in diazepam treated and control animals

Free, protein bound and non-protein bound sulfhydryl content was estimated by the method of Sedlack & Lindsay (1968).

(a) Total SH Content

To 0.5 ml aliquot of the smaple 1.5 ml of 0.2 M tris buffer (pH 8.2) and 0.1 ml of 0.01 M 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in methanol were added. The volume was made upto 10 ml with absolute methanol. Reagent blank (without sample) and sample blank (without DTNB) were also prepared. Colour was developed with mild stirring for 30 mins, after which the samples were centrifugated at 25,00 rpm for 10 minutes and read at 412 nm.

(b) Non-protein -SH content

To 0.8 ml aliquot equal volume of TCA (10%) was added. After shaking it for 10-15 mins, it was centrifuged. To 1 ml supernatant, 2 ml of 0.4 M Tris buffer (pH 8.9) and 0.05 ml DTNB were added and after shaking it vigorously read at 412 nm.

Measurement of glutathione reductase activity

Glutathione reductase activity was measured by the method of Carlberg and Mannervik (1985). To 1 ml cuvette, 0.5 ml of 0.2 M (pH 7.0) potassium phosphate buffer containing 2 mM EDTA at 30 °C, 50 ul NADPH, (2 mM in 10 mM tris -HCl), 50 ul oxidized glutathione, GSSG (20 mM) and a volume of deionized water giving a final total volume of 1 ml was added. The reaction was initiated by the addition of enzyme to the cuvette and the decrease in absorbance, due to oxidation of NADPH followed spectrophotometrically at 340 nm.

$NADPH + H^+ + GSSG \text{ ------> } NADP^+ + 2GSH$

Protein estimation

Protein was estimated in trichloroacetic acid precipitate form all the fractions according to the method of Lowry et al (1951). Bovine serum albumin was used as standard. All spectrophotometric measurements were done on Milton Spectronic 1001 UV/Vis spectrophotometer at constant temperature (25 ± 2 °C).

Statistical analysis

In each group, estimations were done in separate animals, and the statistical evaluations of diazepam-induced alterations in peroxidation, enzyme activity and non enzymatic antioxidants were done by student's `t'test (Fisher, 1950), taking a p value of <0.005 as significant.

RESULTS

To test the role of free radical processes, in the manifestation of effects of diazepam, lipid peroxidation was followed in brain regions of diazepam treated and untreated animals. table1 shows the thiobarbituric acid reactive substance (TBARS) formation in cerebral cortex (CB) cerebellum (CBL) and brain stem (BS) of untreated rats and animals treated with diazepam i.p. and killed after one hour . As evident from the data, a dose of 3 mg/kg body weight, wihich is just 1% of the LD_{50} dose could cause a decrease in peroxidative decomposition of polyunsaturated fatty acids of all the three regions. The malonaldehyde formed in cerebral cortex was 79% of that of control whereas the values obtained for CBL & BS were 85% ($P<0.01$) and 67.5% ($P<0.001$) *of control. Decrease in lipid peroxide formation was also observed at lower (1.5mg/kg* body weight) and higher doses. At lower dose, the changes observed in CB & CBL were similar to that observed at 3 mg/kg dose, whereas in BS, MDA formed was 79.8% of that of coresponding control. This decrease was statistically significant in all the three regions. However, at higher dose i.e., 6 mg/kg, there was further reduction in the TBARS formation being 73.17% in CB, 76.08% in CBL and 48.82% in BS as

Table 1: TBARS formation in different regions of rat brain after single administration of diazepam

	TBARS (nmoles MDA/mg protein/hr)		
Treatment	**CB**	**CBL**	**BS**
Untreated	0.816±0.026	1.049±0.049	0.887±0.023
1.5 mg/kg D (A)	0.664±0.025[a]	0.805±0.047[a]	0.707±0.034[a]
3.0 mg/kg D (B)	0.662±0.091	0.891±0.070[c]	0.599±0.042[a]
6.0 mg/kg D (C)	0.597±0.033[a]	0.798±0.019[a]	0.433±0.058[a]

Values are arithmetic mean ± S.D. of six determinations in each case.

a: $p < 0.001$; b: $p < 0.02$; c: $p < 0.01$

compared to their corresponding controls and were highly significant ($p<0.001$). The changes observed in the brain stem regions were were highly significant ($p<0.001$). The changes observed in the brain stem region were highly significant with all the 3 doses tested and of a 51.18% decrease in lipid peroxidation was observed with the 6 mg dose. For further experiments, 3 mg/kg dose was chosen, which showed moderate changes and differed in its response in all the regions of brain examined since the effects of the tranquilizers is reversible, it was of interest to see whether the oxidative stress was transient or persisting. Time course of the TBARS formation was follwed in control, group I (sacrified after 1 hour of dose) and group II animals (sacrified after 18 hours of dose). For this purpose, aliquots were drawn form the assay systems at 0 time, 30 minutes, 60 minutes and 120 minutes and MDA formed at that particulan time was measured in all the groups of animals compared. Fig. 1 shows a comparative account of MDA formation in cerebral cortex of control and two groups of diazepam treated animals at different time intervals. As is evident from the data, the 0 time value of MDA formation was63.7% higher in group I rats. This is an index of the *in vivo* lipid peroxides already formed due to diazepam administration. This was not seen in the case of grp. II animals showing values comparable to controls rats. At 30 minutes, MDA formed in control and grp. I animals was comparable whereas grp. II animals showed a decrease in MDA formed being 53.5% lower than untreated rats. At 1 hour grp. I showed a little higher value whereas grp. II showed lower TBARS formation, but taking into consideration the zero time value, the actual rate of MDA formation was lower in grp. I animals than controls. After two hours of incubation there was reduced lipid peroxidation in grp. I and and grp. II animals being 39.23% and 57.7% lower than corresponding controls respectively

Fig. 2 shows the rate of lipid peroxidation in cerebellum. In this case also at zero time TBARS formed in grp. I rats was 55% higher then untreated rats. Again deducting the values obtained at 0 time there was a progressive decrease in TBARS formation in grp. I and grp. II animals.At one hour this decrease was 31.48% in grp. I and 24.7% in grp. II rats, whereas at 2 hrs it was 25.25% and 28% respectively. In the brain stem region (Fig 3) however, there was not much difference in the zero time value of grp. I animals as compared to controls, whereas grp. II animals showed a 60% lower MDA formation at zero time .This reduction in lipid peroxide formation was maintained even at 30 minutes being 52% and 71.6% respectively in group I and group II rats. This

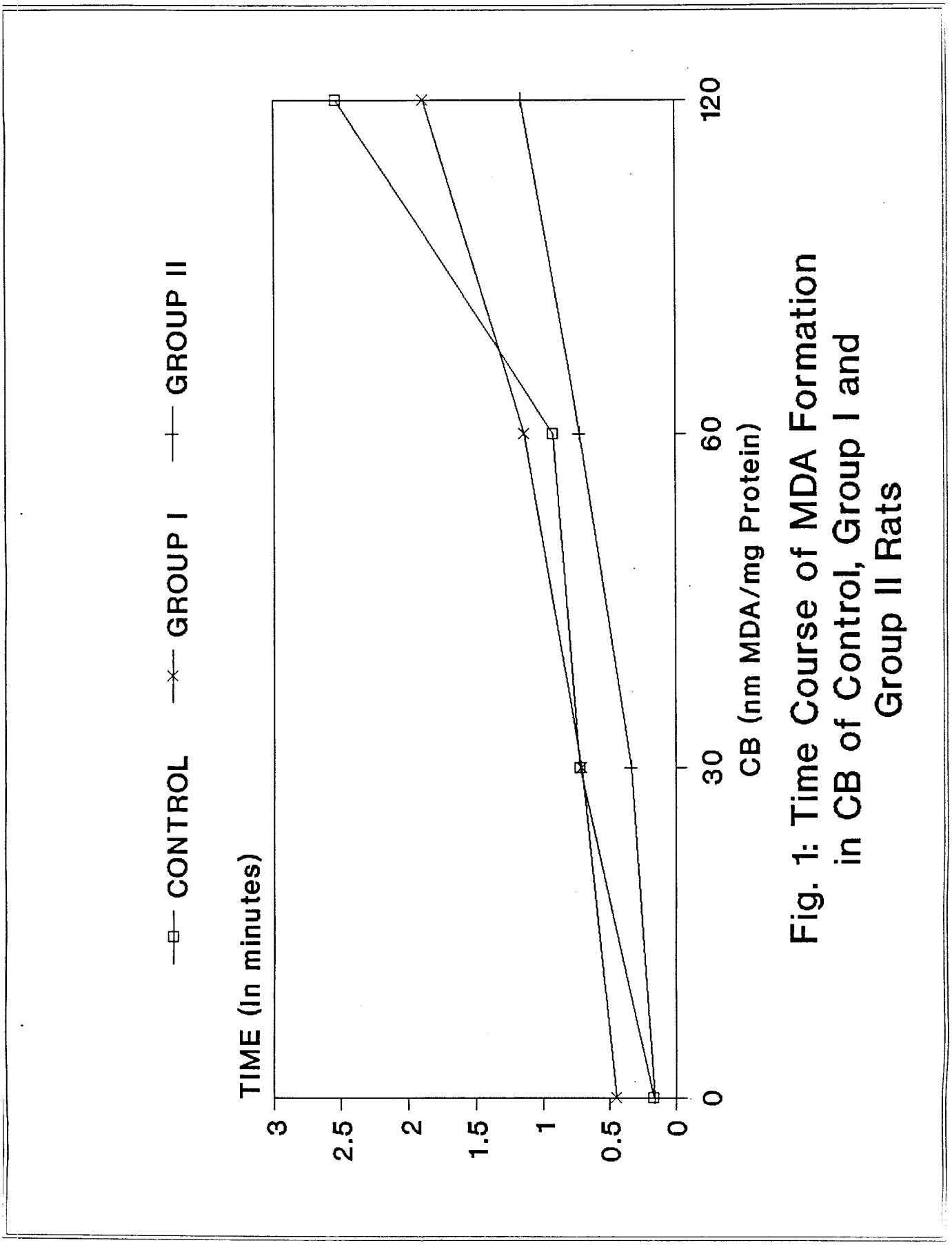


Fig. 1: Time Course of MDA Formation in CB of Control, Group I and Group II Rats

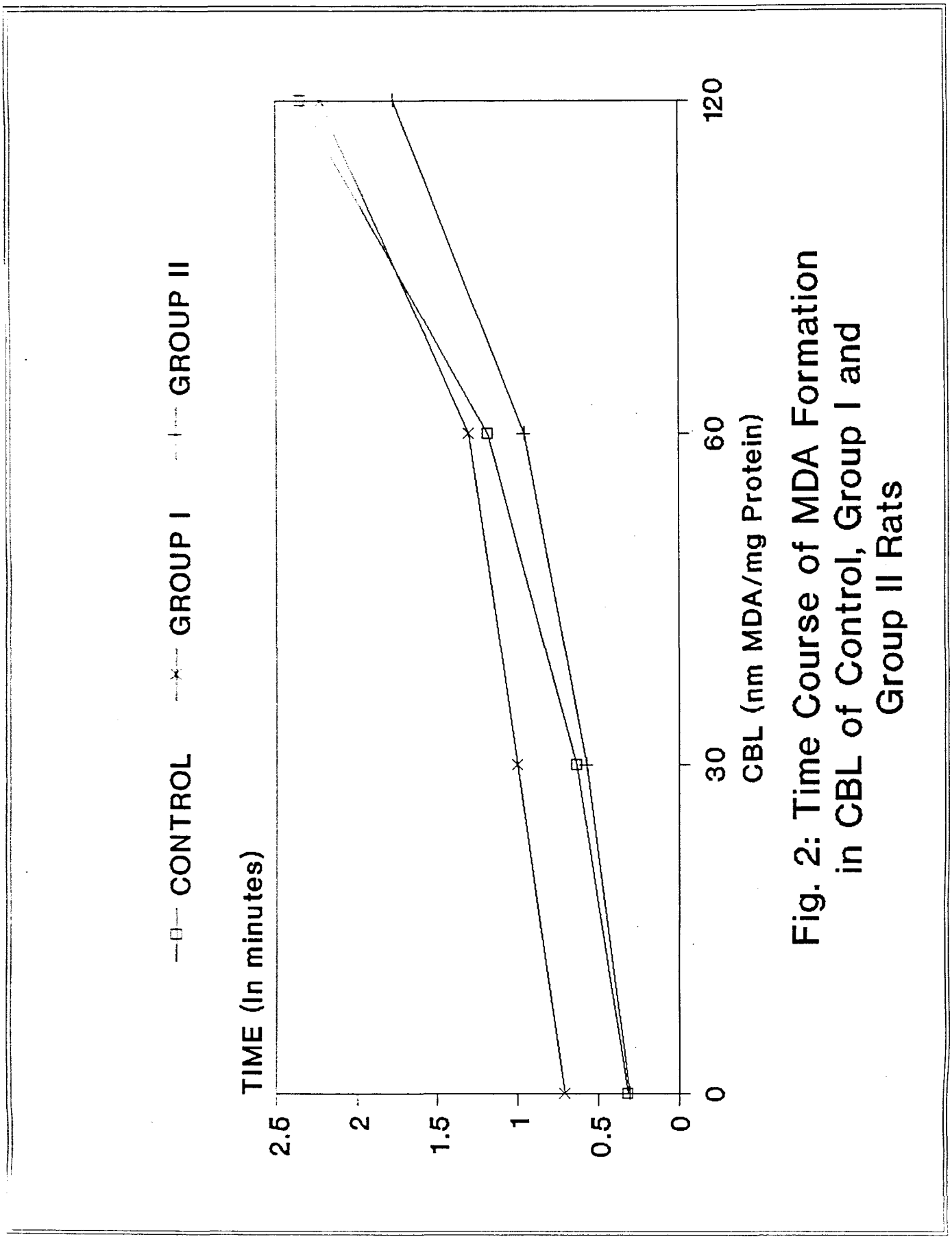


Fig. 2: Time Course of MDA Formation in CBL of Control, Group I and Group II Rats

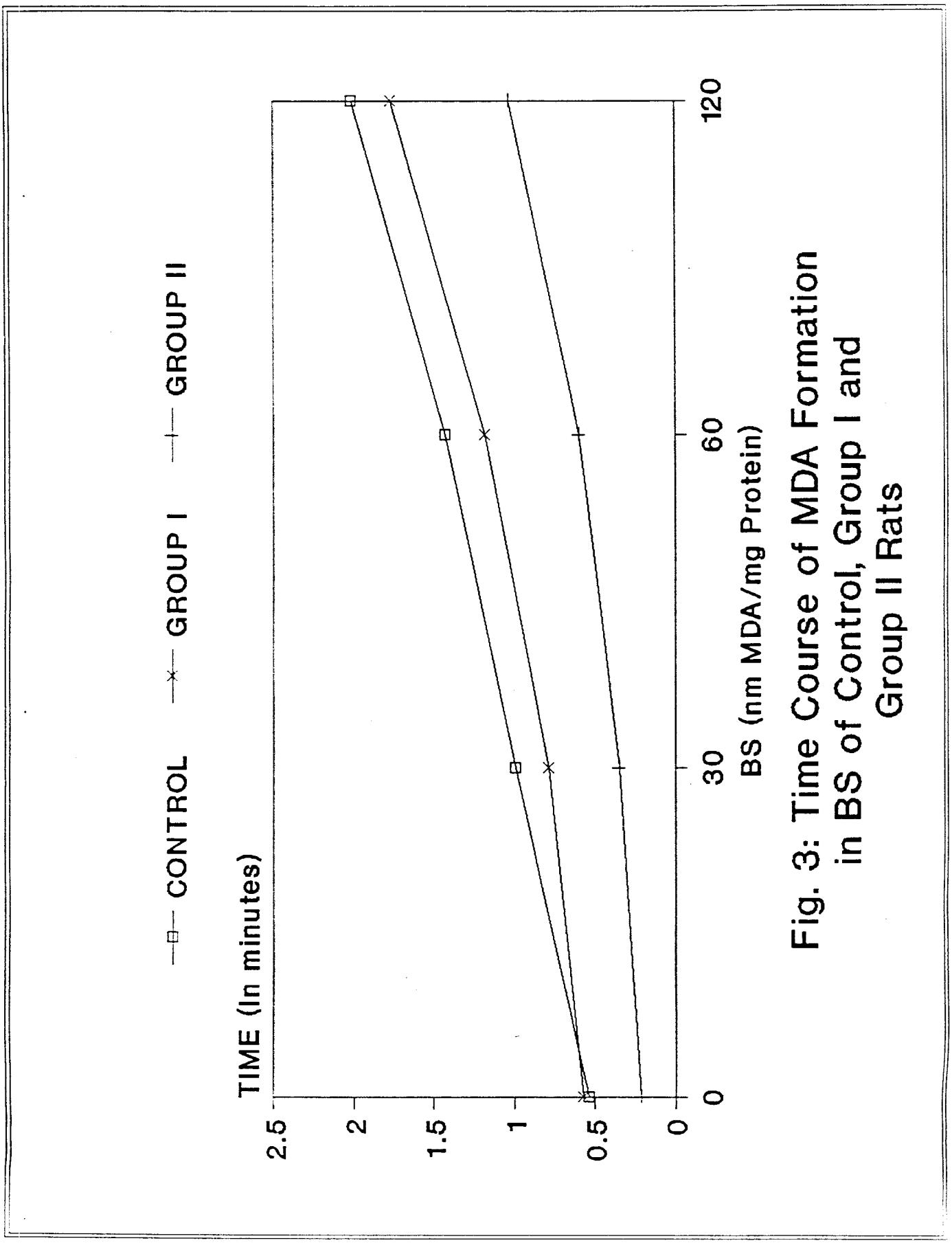


Fig. 3: Time Course of MDA Formation in BS of Control, Group I and Group II Rats

difference in the TBARS formation in diazepam treated animals was however reduced at 1 hour being 31.5% and 57.5% lower than corresponding control. This was even further reduced after 2 hrs with grp. I animals showing only 19% lower TBARS formation and grp. II animals showing 44.7% lower values. The data indicates towards an early effect of diazepam administration on the rate of lipid peroxidation. Maximum lowering of TBARS formation was seen at 30 minutes which was progressively reduced till 2 hrs, since prooxidative process could differ in various cellular compartments. Subcellular fractionation of the homogenates was done and TBARS formation was followed in mitochondria of CB, CBL and BS regions (Fig. 4). As is evident from the data, an entirely different picture emerged in this organelle as compared to whole homogenates of brain regions. In the mitochondria of CBL there was 21% enhancement ($p < 0.02$) as compared to its corresponding control. Highest enhancement was observed in BS being 107% of the control which is highly significant ($p < 0.001$). A 30% enhancement in lipid peroxidation was observed in the mitochondria of cerebral cortex ($p < 0.001$). Hence, mitochondria showed a specific response which was also organelle specific.

In the cytosolic fraction, fig. 5, a decrease in MDA formation was observed in carebral cortex which was statistically highly significant ($P<0.001$). Cerebellum showed a 49% enhancement in lipid peroxidation which was again significant ($p< 0.001$). The decrease observed in BS region was not significant. Thus, in this fraction also, a region specific response was observed due to diazepam administration.

Effect of diazepam on the antioxidant status of different brain regions was also followed Fig. 6 shows the effect of diazepam on mitochondrial superoxide dismutase (Mn-SOD). A decrease in the activity of Mn-SOD was observed in all the 3 regions. The enzyme activity was decrease by 38% in CB. Whereas in BS lowering of the enzyme acitivity was maximum, being 52% ($p<0.001$). The decrease observed in the Mn-SOD of CBL was found to be non-significant. Fig. 7 shows the Mn-SOD activity in group II animals and it is clear from the data that the lowering of enzyme activity was fully recovered after 18 hours. In cerebellum however, a significant ($p< 0.001$) increase of the enzyme activity was seen after 18 hrs which could be due to a better response against free radical stress in this region.

Cu-Zn superoxide dismutase isozymes also showed a similar response to diazepam administration. As is evident from fig. 8 cerebral cortex and brain stem showed a significant decrease ($p< 0.001$) in enzyme activity being 30% lower than the

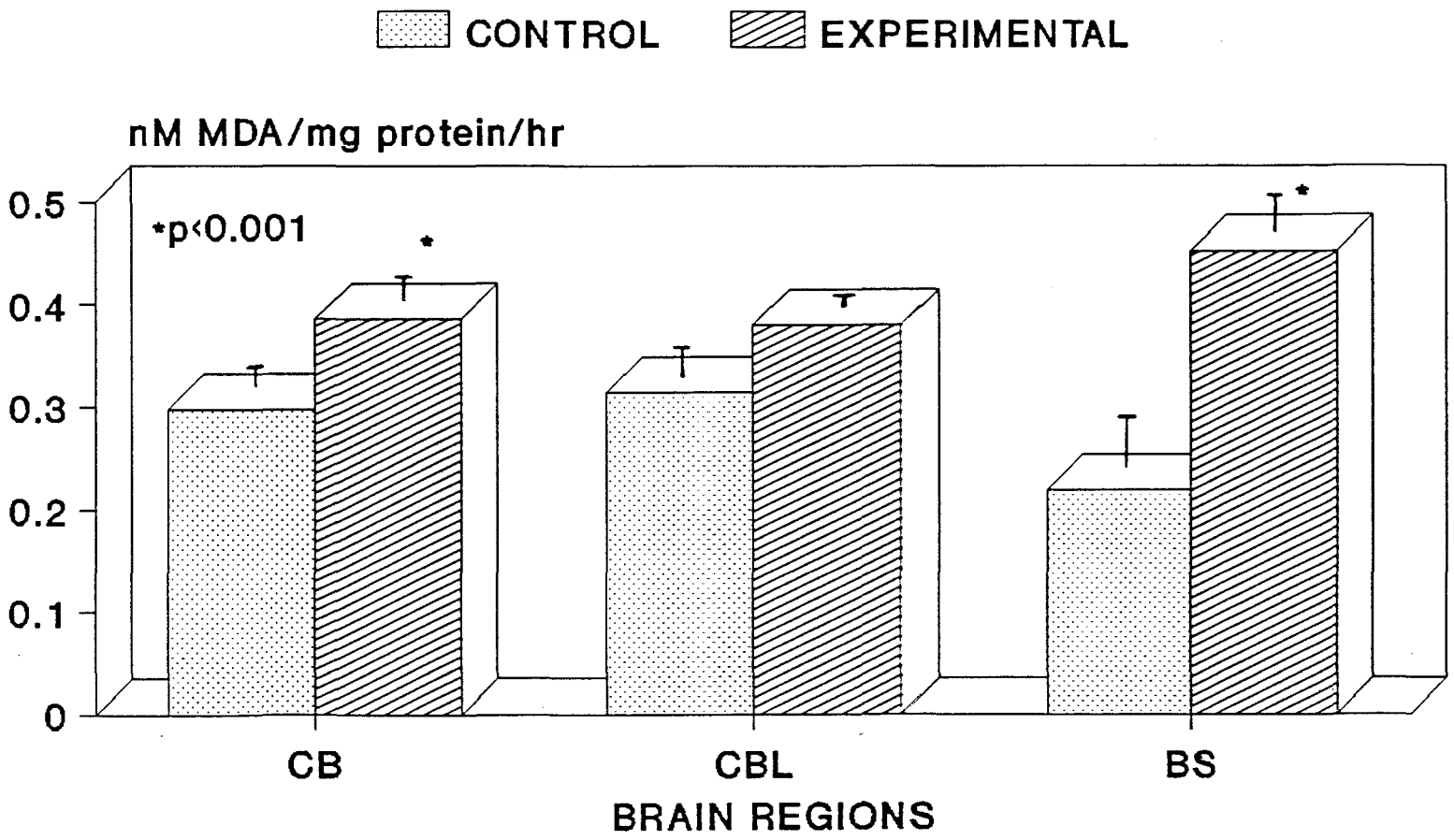


Fig. 4: Effect of Diazepam on TBARS Formation in Mitochondrial Fractions in Group I Rats

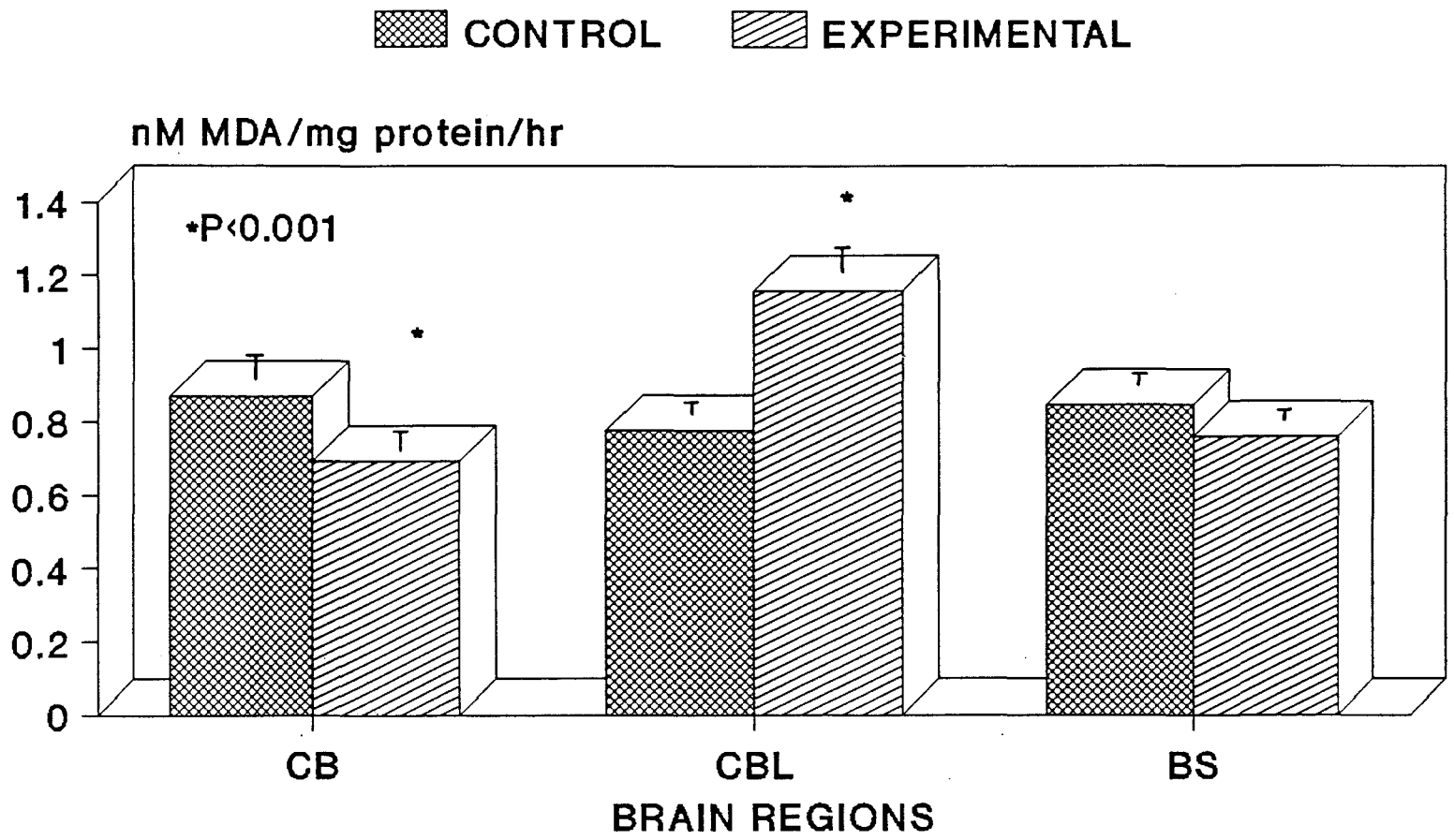


Fig. 5: Effect of Diazepam on TBARS Formation in Cytosolic Fractions in Group I Rats

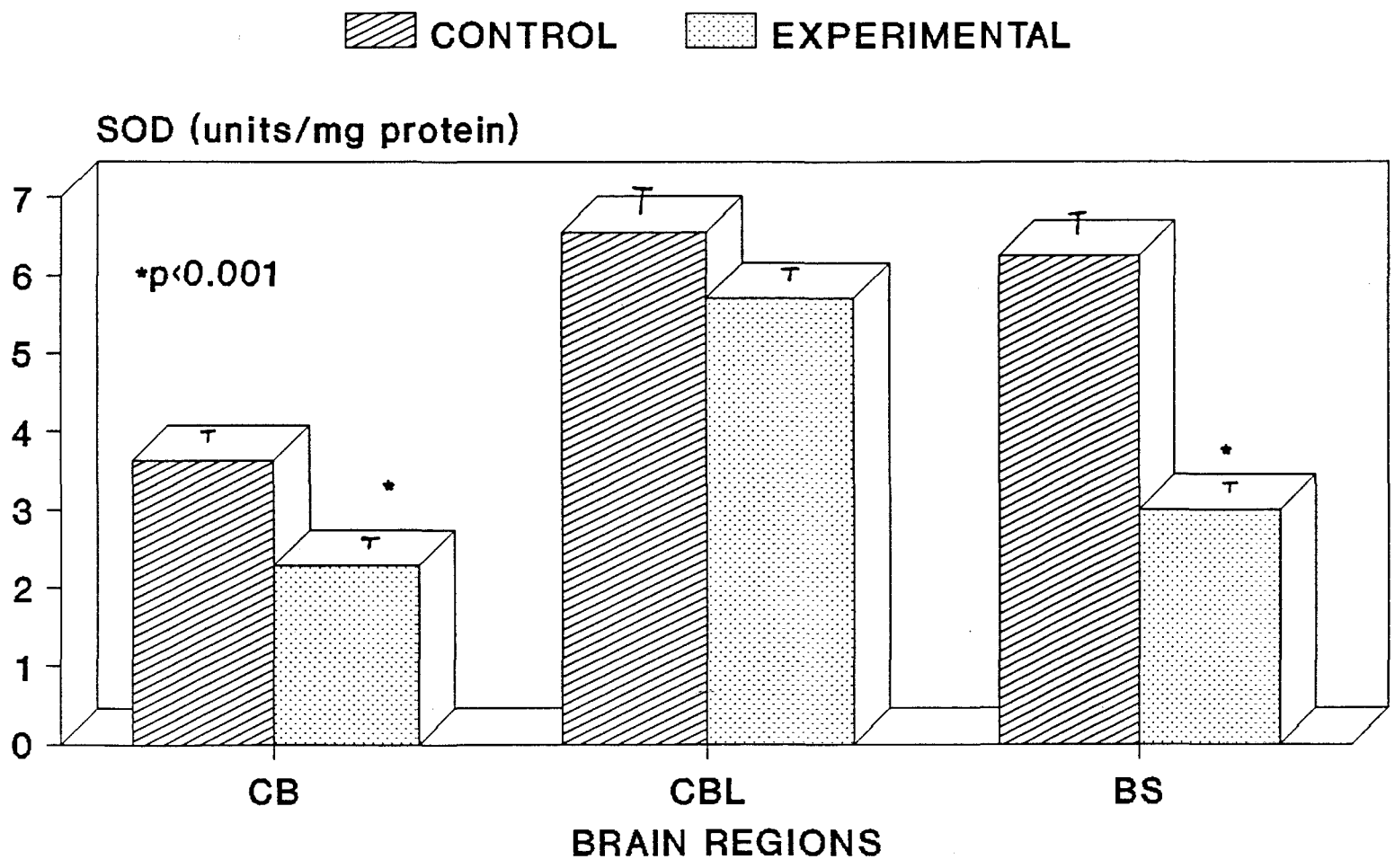


Fig. 6: Effect of Diazepam on Mn-SOD of Group I Rats

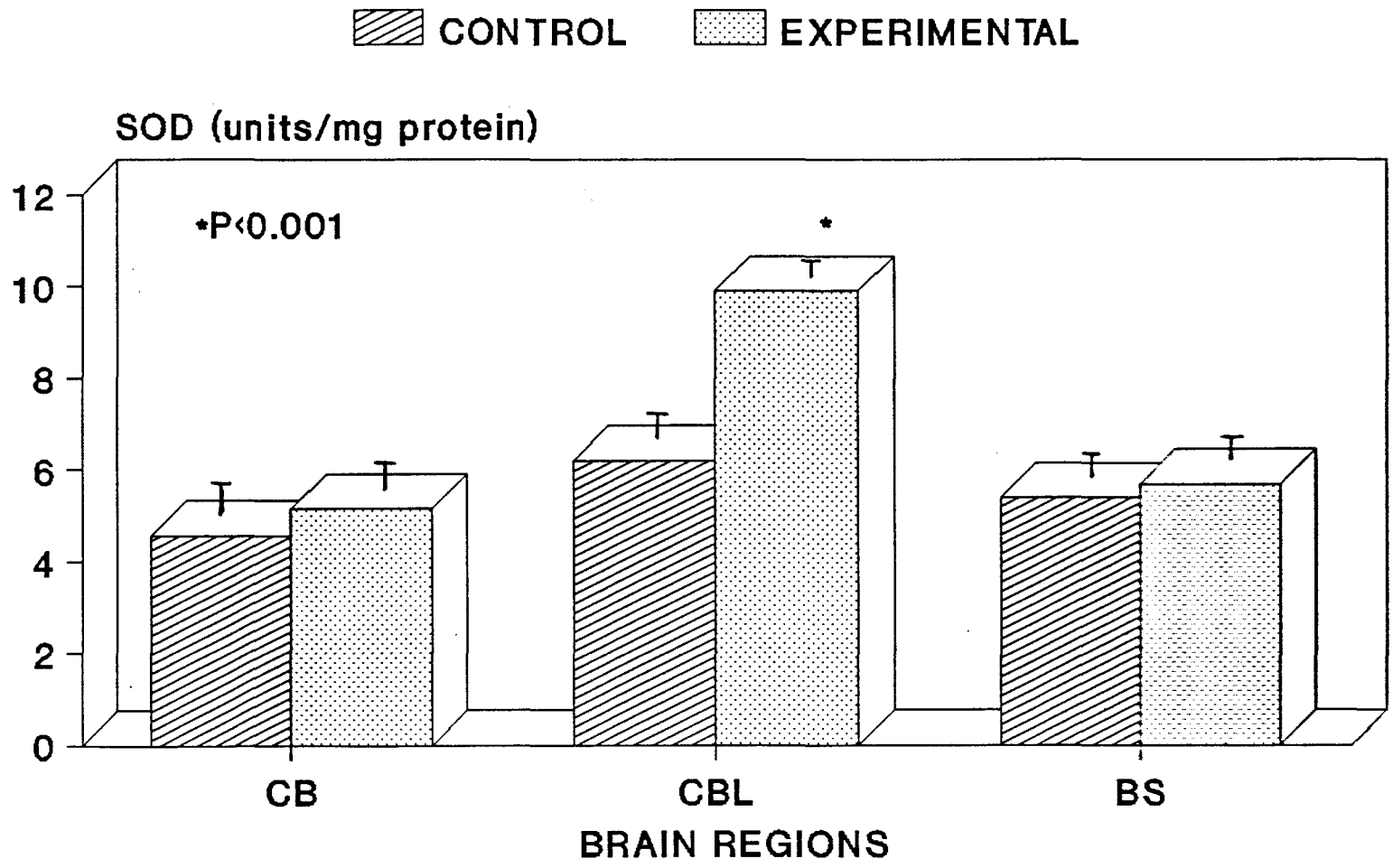


Fig. 7: Changes due to Diazepam on Mn-SOD of Group II Animals

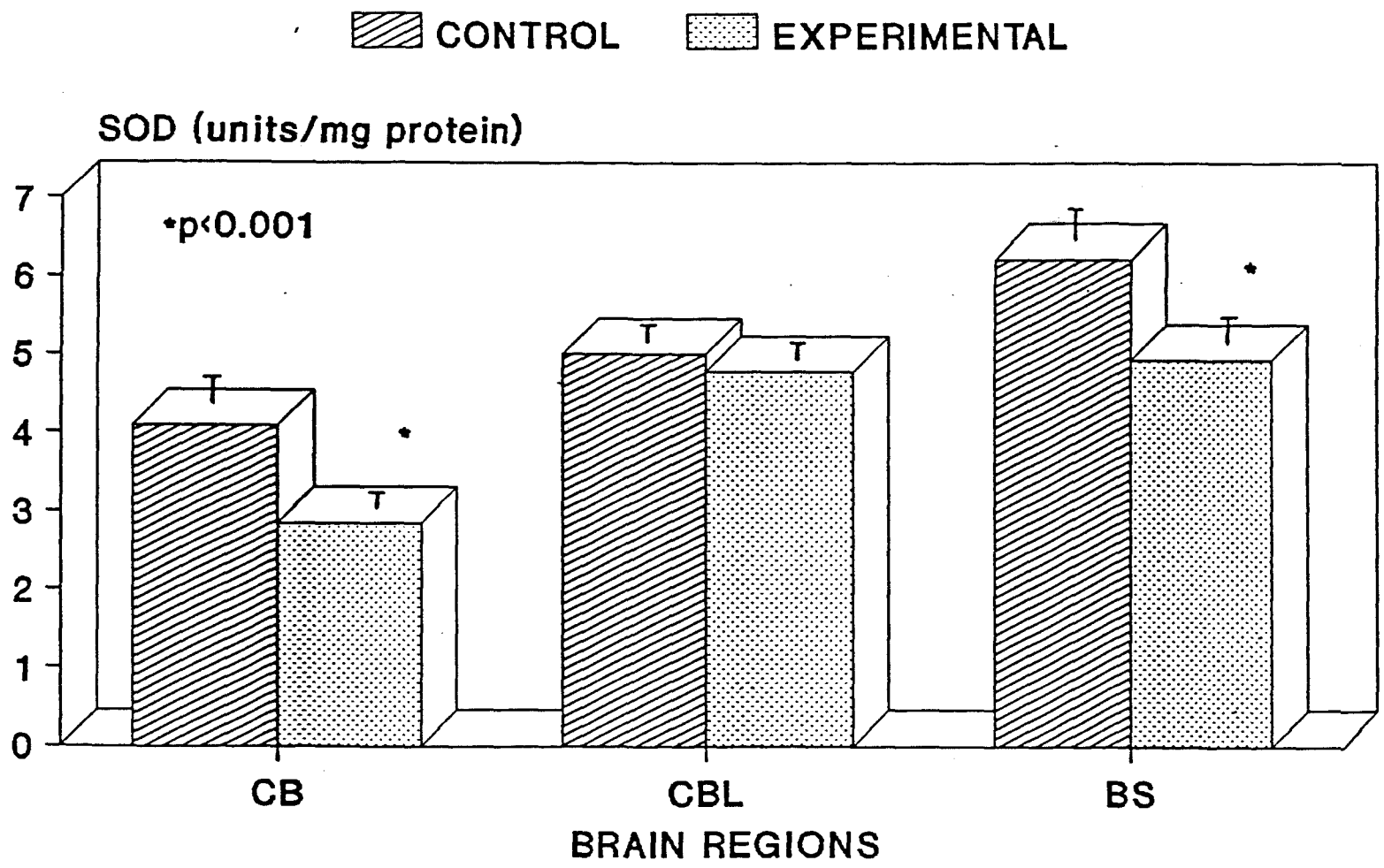


Fig. 8: Effect of Diazepam on Cu-Zn SOD in Group I Rats

corresponding control in the case of CB. The lowering of CuZn SOD activity in CBL was statistically non-significant. A trend towards recovery of the enzyme activity was evident effect in lue of diazepam administration (Fig.9). In CB a significant increase in Cu Zn SOD was seen, indicating towards a good response for an onslaught of free radicals. Enzyme activity in the CBL of grp. II animals was also increased as compared to controls. In BS, the activity was a little lower than control but this difference was non-significant.

Effect if any, on the total thiol content of the brain regions was also observed, since thiol content plays a very important role by supplying reducing equivalents and also by counteracting the onslaught of free radicals. The data in table 2 shows the total- SH content in untreated and group I animals. No significant change was observed in total thiol content in any of the 3 regions However, free thiol which comprises mainly of GSH (reduced glutathione (Table 3) showed depletion in all the regions of rat brain being highly significant in CBL & BS ($p< 0.001$) A 39.8% depletion of free thiols was observed in cerebellun whereas in BS a 50% depletion was observed in the treated animals. In cerebral cortex the depletion was only 7.2% and was not significant. Highest effect was observed in the brain stem region. On following the thiol level in gr II animals, no significant change in the total thiol content was observed in any region (Table 4). However, the free thiol content in grp. II animals showed significant decrease in all the brain regions even after 18 hours of diazepam administration. In CBL (Table 5) the decrease in free thiol content was about 29% whereas in BS it was 27.2%.The data clearly shows that diazepam administration affects the nonenzymatic antioxidants as well and causes an imbalance in reducing equivalents of the cell. The effect was quite pronounced and even after a gap of 18 hrs though there was some regeneration of free thiols but it did not return back to normal levels.

Activity of glutathione reductase, the key enzymes involved in the regeneration of GSH from GSG, was also estimated under the same treatment schedule in mitochondrial and cytosolic fractions. Table 6 shows mitochondrial glutathione reductase activity in group I animals. A significant ($p < 0.001$) decrease in the enzyme activity was observed in the mitochondria of CBL sand BS regions of grp. I animals. In CBL the activity was 72.41% and in BS it was 59.1% of that of respective controls. In the mitochondrial fraction of cerebral cortex although there was a little decrease in the activity but it was not significant. Similar trend was observed in the cytosolic fractions

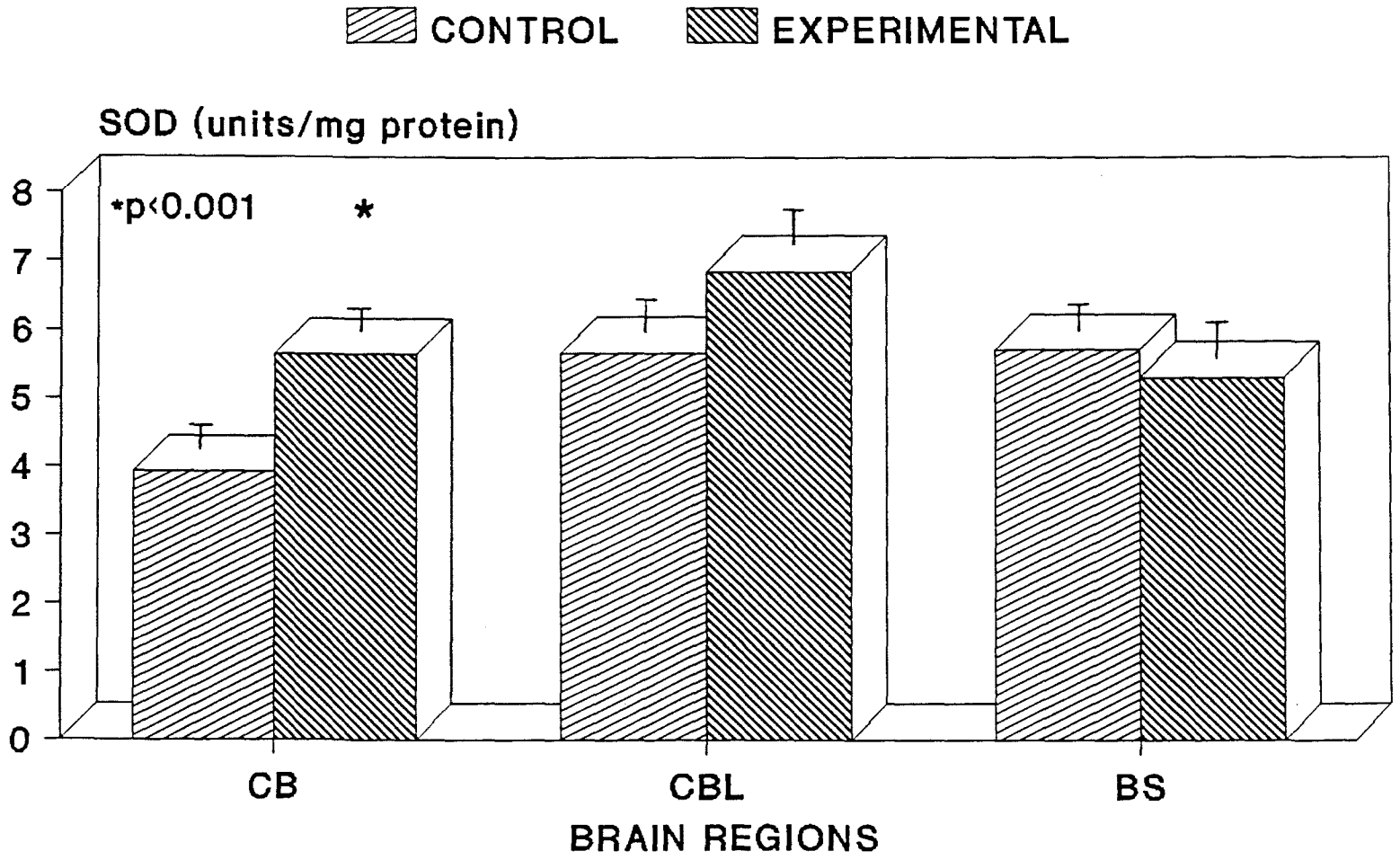


Fig. 9: Changes due to Diazepam on Cu-Zn SOD of Group II Animals

Table 2: Effect of diazepam on total -SH content in different brain regions of group I rats

	Total -SH content (umoles -SH/g fresh tissue wt.)	
	Control	**Treated**
CB	26.86±0.982	26.143±1.339
CBL	19.312±1.488	18.675±1.517
BS	20.531±1.309	19.779±0.854

Values are arithmetic mean ± S.D. of six determinations in each case.

Table 3: Free Thiol content in different brain regions of group I rats

	Free -SH content (umoles -SH/g fresh tissue wt.)	
	Control	**Treated**
CB	3.011±0.199	2.792±0.379
CBL	1.344±0.102	0.809 ±0.071[a]
BS	1.266±0.023	0.631 ±0.021[a]

a: $p < 0.001$

Values are arithmetic mean ± S.D. of six determinations in each case.

Table 4: Effect of diazepam on total -SH content in different brain regions of group II rats

	Total -SH content (umoles -SH/g fresh tissue wt.)	
	Control	**Treated**
CB	21.965±1.692	20.040±0.815
CBL	21.586±2.487	20.445±1.288
BS	20.075±1.042	20.850±0.410

Values are arithmetic mean ± S.D. of six determinations in each case.

Table 5: Free thiol content of different brain regions in group II rats

	Free -SH content (umoles -SH/g fresh tissue wt.)	
	Control	**Treated**
CB	2.477±0.044	2.097±0.055[a]
CBL	1.292±0.0658	0.919±0.021[a]
BS	1.187±0.097	0.864±0.057[a]

a: $p < 0.001$.

Values are arithmetic mean ± S.D. of six determinations in each case.

Table 6: Glutathione reductase activity in the mitochondria of different brain regions in group I rats

	Glutathione reductase activity (unit x 10-3/min/mg protein)	
Mitochondria	**Control**	**Treated**
CB	19.85±2.540	18.346±1.526
CBL	21.29±1.855	15.416±0.701[a]
BS	18.743±0.986	11.076±0.061[a]

a: $p < 0.001$

Values are arithmetic mean ± S.D. of six determinations in each case.

Table 7: Glutathione reductase activity in the cytosol of different brain regions in group I rats

	Glutathione reductase activity (unit x 10^{-3}/min/mg protein)	
Cytosolic Fractions	**Control**	**Treated**
CB	35.706±0.781	31.020±2.277[b]
CBL	39.955±1.034	37.520±0.895
BS	31.377±0.676	29.720±0.971[b]

b: $p < 0.02$

Values are arithmetic mean ± S.D. of six determinations in each case.

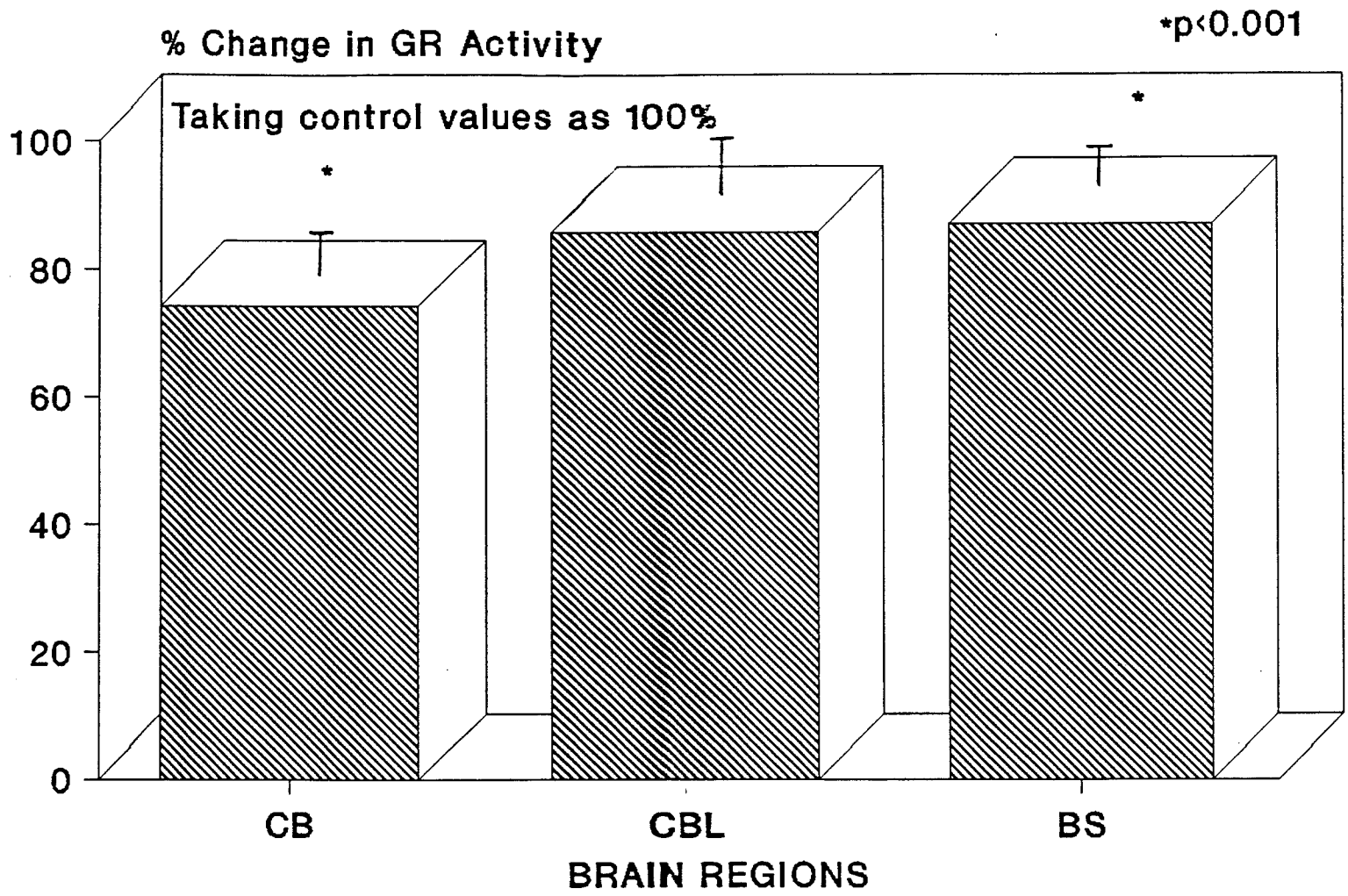


Fig. 10: Percent Change in GR Activity of Group II in Mitochondrial Fractions

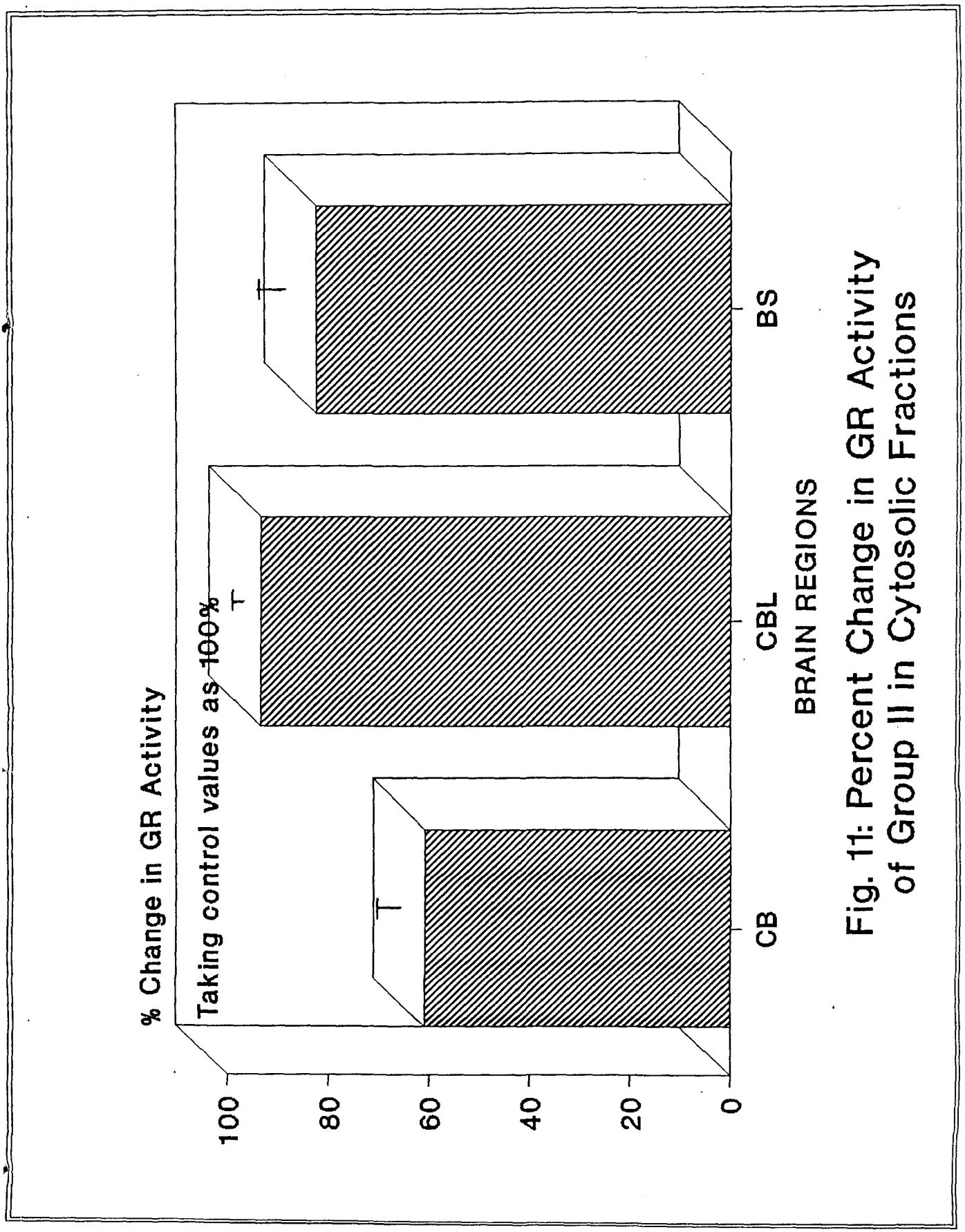


Fig. 11: Percent Change in GR Activity of Group II in Cytosolic Fractions

(Table 7) also where moderately significant ($p < 0.02$) changes were observed in CB and BS being 68.8% and 94.72% of that of corresponding controls. In CBL, the activity was 94% of that of control. Significant decrease in the glutathione reductase activity in the CBL and BS supports the data for free thiol depletion in these regions on diazepam treatment.

In group II animals, a trend towards recovery of glutathione reductase activity was observed in mitochondria of all the regions, as is evident from figure 10. In cerebellum, the activity increased from 72.41% in group I animals to 85.79% in group II animals. Similarly, in brain stem it, increased from 59.1% in group I to 87.05% in group II showing a 47.2% recovery in the activity in this region. Figure 11 shows the changes in the glutathione reductase activity in the cytosolic fractions. In CB and BS both, a further decrease in the activity was observed. In CB, the activity was 60.91% and in BS it was 82.49% of control. In CBL a 7% decrease in activity was observed. The data thus indicates that, in mitochondria after 18 hrs the effect of diazepam on glutathione reductase activity starts vanishing whereas in cytosolic fraction the effect of diazepam remains and in fact a further decrease in enzyme activity was observed.

The results indicate the involvement of free radical processes and modulation of enzymatic and non-enzymatic antioxidant defenses during the hypno-sedative effects of diazepam. The results clearly show that the responses not only differ in the different regions of rat brain but are also organelle specific, showing a different trend in mitochondria and cytosol.

DISCUSSION

Diazepam is a drug widely used clinically and with the growing preference for benzodiazepines in stress management its use has increased in recent times. It is widely prescribed as a sedative agent apart from its use as an anticonvulsant, anxiolytic agent and muscle relaxant. It is generally considered a very safe drug. A few deaths in humans have been reported at doses greater than 700 mg (Morselli, 1977). Cardiovascular and respiratory depression may occur after intravenous administration of diazepam. Its acute LD_{50} in rats is 710 mg/kg when administered orally, whereas intraperitoneally it is 300 mg/kg (Gilman et al., 1991). The immediate prooxidative changes caused by single injection of diazepam observed here is due to its quick distributin and delivery to the brain. Since the lipid solubility of diazepam is 99%, its quick effect on membrane lipids after 1 hour of treatment is understandable. The results (Table 1) show that in the tissue homogenate, lipid peroxidation was inhibited as compared to untreated rats and it was dose dependent and region specific. Diazepam related inhibition of TBARS formation was found to be maximum in brain stem where a 51.18% decrease in lipid peroxidation was observed.

During the course of lipid peroxidation it was observed that the zero time value for diazepam treated group I rats differed in CB and CBL being 63.7% and 55% higher than

corresponding controls. This indicates towards high lipid peroxide formation immediately after diazepam administration in these regions *in vivo*. In CB after 2 hours of incubation there was still inhibition of LPO which was more pronounced in group II animals. In the brain stem region, initially the level of MDA was similar in control and group I animals but group II animals showed lower level of MDA formed. Maximum reduction in TBARS formation was seen at 30 minutes showing that effect of diazepam is pronounced at initial stages of lipid peorxidation.

This response differed at the subcellular level. Mitochondria CB, CBL and BS (Figure 4) showed higher peroxidation of polyunsaturated fatty acids after diazepam administration. Highest LPO was observed in the brain stem mitochondria showing 2 fold increase. Any inhibition caused by the other constituents of the cell, which gets removed during subcellular fractionation needs to be explored. The regional and subcellular distribution and turn over of antioxidant defenses could also account for the variation in effects. Another point of interest was that Mn-SOD activity of BS was also significantly decreased ($p < 0.001$) being 52% lower than the corresponding control. In group II animals, however, the Mn-SOD activity came back to normal level, even showing an increase in CBL.

In the cytosolic fraction (Fig. 5) a decrease in LPO was observed except in CBL. Simultaneously an inhibition of Cu-Zn SOD activity was also observed which was recovered after 18 hrs.

Earlier work from this laboratory and literature reports (Castilho, 1995) show that mitochondria are an important cellular site for both the generation of oxygen radicals and oxidative damage (Mehrotra *et al.*, 1991; 1993; Kakkar *et al.*, 1992a, 1995, 1996). Also since mitochondria posses peripheral benzodiazepine receptors on the outer membranes (Krueger and Papadopoulous, *et al.*, 1992) binding of diazepam to these receptors and thus facilitating peroxidative decomposition of membrane lipids cannot be ruled out. Lowering of Mn-SOD in diazepam treated rats may also facilitate free radicals reaching the PUFA of membranes without being counteracted. The results show that the effects were more pronounced in mitochondria as compared to cytosol, presumably due to higher role of active oxygen formation and lower antioxidants.

In the recent past many drugs have been found to produce $O_2^{\cdot}$ and H_2O_2 during their metabolism (Farber, 1986) and the toxicity of these drugs has been attributed to the generation of free radicals and resultant oxidative stress rather than due to cellular

interaction of the reactive metabolites derived from the parent compound. Availability of nucleophile GSH in the cellular environment to counteract electrophilic metabolites of xenobiotics formed through bioactivation, may sometimes prove to be the deciding factor. The results also indicate depletion of free thiols (Table 3) (which mainly comprises of GSH), due to diazepam which was most pronounced (50%) in brain stem region. Cerebellum also showed 39.8% depletion. The maximum depletion of GSH in the brain stem region correlates well with the data for lipid peroxidation being highest in this region. Also, Mn-SOD was found to be lowest in brain stem after diazepam administration. Even in group II animals there was significant decrease in GSH in all the regions. In CBL, the GSH depletion recovered from 39.8% to 29% and in BS from 50% to 27.2% but could not attain the normal levels.

The data shows that though there was no significant change in the total thiol content in brain regions due to diazepam, a significant depletion of free thiols was observed in cerebellum and brain stem. The possibility of protein thiols forming cross- linked protein aggregates cannot be ruled out at this stage. Formation of GSH-protein mixed disulfides alongwith GSH depletion have been reported during the metabolism of menadione (DiMonte *et al.*, 1984). Depletion of GSH on one hand affects the detoxification of toxic metabolites, being the substrate of glutathione-S-transferase (Wyatt t al., 1996) and on the other hand renders the cells more vulnerable to oxidative free radical damage. In earlier studies from this laboratory GSH depletion was observed in aniline exposed animals (Kakkar *et al.*, 1992). Differences in the regional distribution of thiols and other antioxidant enzymes plays a major role in regional differences in capacity for metabolic processing of both endogenous compounds and xenobiotics (Philbert *et al.*, 1995).

Lowering of glutathione reductase activity due to diazepam treatment further supports the results and confirms the involvement of oxidative processes during the hypnosedative effects of diazepam. Highest inhibition of this *enzyme* activity was found to be in the brain stem region being 40.9% followed by cerebellum where the decrease was 27.59% (Table 6). This could explain the depletion of free thiols being highest in the brain stem region as the conversion of GSSG to GSH is also affected the most in this region followed by cerebellum. Moderately significant decrease in GR activity was observed in cytosols of these regions (Table 7). In group II animals, a trend towards recovery of the activity was observed. In BS region mitochondrial glutathione reductase activity showed 47.2% recovery (Fig. 10) but in the cytosol no recovery was seen.

There are literature reports of tremendous regional heterogeniety in brain and each region displays specialized and selective functions (Ravindranath *et al.*, 1995). This data also shows that there is regional heterogeniety in the distribution of enzymatic and non-enzymatic antioxidants and thus differences in the response towards any free radical onslaught. The differences observed in the lipid peroxidation in the regions and also at the subcellular level gives a new insight into the free radical attack due to diazepam. Courtiere *et al.* (1995) have recently reported that peripheral type benzodiazepine receptors of liver are more affected by peroxidative events than central type BZ receptors of the cereberal cortex. Apart from this a point of interest that emerged from these results was the differential response at the subcellular level. Mitochondria were found to be more affected by diazepam. The higher TBARS formation in the mitochondria of BS alongwith 2 fold decrease in Mn-SOD activity, depletion of free thiols bringing it down to half the level of normal alongwith decrease in GR activity being maximum in this region suggests that BS is affected more at the mitochondrial level as compared to CB and CBL regions of brain. Lower antioxidant defenses leave the cells quite vulnerable to assualt by active oxygen species. It has been reported earlier that there is a coordination between the $O_2^{\cdot}$ radical production and thiol recycling (Kehrer and Lund, 1994). During reductive stress i.e. when the redox-cycling of thiols gets disturbed, mitochondria become a good source of reactive oxygen species (Dawson *et al.*, 1993).

Lipid peroxidation affects the cell membranes and organelle membranes, leading to a compromise in the impermeability of membranes, disturbance in the ion fluxes, affects the membrane bound proteins including the ion pumps, which can lead to disturbed cellular Ca^{2+} and thiol homeostasis and induction of degradative processes and ultimate damage. It would be of interest to see the effect of diazepam on Ca^{2+} related functions especially in mitochondria for future studies. Thus the data indicates towards an involvement of free radical related processes and modulation of antioxidant defenses during hypno sedative effects of diazepam. Since earlier work from our laboratory has shown an interrelation between deregulation of Ca^{2+} cycles and oxygen radical mediated membrane changes (Kakkar *et al.*, 1992a, 1995, 1996) any involvement of the central unspecific pathway in toxicity i.e. xenobiotics ------> free radicals --------> membrane damage -------> altered calcium functions ---------> diversity of toxic effects, during diazepam treatment would be be further explored.

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